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Characterization of F3II, a Sarcomatoid Mammary Carcinoma Cell Line Originated From a Clonal Subpopulation of a Mouse Adenocarcinoma

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We characterized a new mammary tumor cell line, F3II, previously established in vitro from a clonal subpopulation of the BALB/c transplantable mammary adenocarcinoma M3, moderately metastatic to lung. The F3II cell line has been passaged >50 times. It has grown as elongated cells adherent to the bottom of the flask. Cytogenetic studies showed that F3II cultures were nearly triploid. Tumor cells expressed fibronectin and showed high levels of cell-surface urokinase, a key protease in invasion and metastasis. F3II cells grew as poorly differentiated, spindle-cell carcinoma tumors (sarcomatoid carcinomas) with a prominent local invasiveness, a high angiogenic response, and a 90–100% incidence of lung metastases when inoculated s.c. into syngeneic mice. Ultrastructural and immunocytochemical analysis revealed characteristic features of carcinomas. Our data suggest that F3II is less differentiated and more aggressive than the original tumor line, supporting the notion that mammary carcinomas are heterogeneous neoplasms and contain subpopulations with diverse biologic behavior. The F3II mouse mammary sarcomatoid carcinoma line is a suitable model to examine antiinvasive, antiangiogenic, and antimetastatic agents.

KEY WORDS: **breast cancer, carcinosarcoma, heterogeneity, urokinase, invasion, metastasis**

INTRODUCTION

A consistent body of evidence suggests that cancerous tumors usually contain subpopulations of cells with different biological properties [1,2]. It is believed that malignant cells are unstable and may undergo frequent changes during progression, thus producing tumors with a mixture of different cell subpopulations [3]. Metastasis is the end result of a sequential, multistep process and constitutes the main problem in cancer management. It has been shown that metastases are not random but result from the selective growth of a specialized subpopulation of cells preexisting within the primary tumor [4].

Proteolytic degradation of the extracellular matrix is an essential step in neovascularization, tumor cell invasion, and metastasis formation [5]. Cancer cells are capable of degrading most components of surrounding tissues via an extracellular proteolytic cascade, including the serine proteases urokinase-type plasminogen activator (uPA) and plasmin and the matrix metalloproteinases [5–7]. In malignant tumors, uPA is secreted as a soluble protein that binds to a high-affinity, cell-surface receptor present on invasive cells, catalyzing the conversion of plasminogen to plasmin. In turn, plasmin degrades fibro-

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nectin, laminin, and other noncollagenous proteins of the extracellular matrix and is able to activate latent collagenases [8,9].

Several mouse systems have been very useful to study the invasive process, as well as the possible role of intratumor heterogeneity in metastasis formation. In addition, well-characterized tumor cell lines are needed for in vitro and in vivo model systems to test the efficacy of antineoplastic agents [10–14].

We are studying the biological implications of intratumor heterogeneity in a transplantable mammary adenocarcinoma spontaneously grown in a BALB/c mouse, designated M3 [15]. In a previous work [16], a total of 32 clonal subpopulations were separated and expanded in vitro from primary tumor cell suspensions prepared from M3 subcutaneous tumors. Most cultured tumor cell subpopulations were composed of epithelioid polyhedric cells, as the original M3 population. However, one clonal subpopulation, named F3II, was composed of spindle-shape cells [16,17].

The purpose of this work was to characterize the new F3II mammary tumor cell line with special reference to the invasive and metastatic abilities and the expression of fibronectin and uPA. In vitro and in vivo data suggest that F3II is less differentiated and more aggressive than the original tumor line.

MATERIALS AND METHODS

Cell Lines and Cell Culture

F3II and M3 cell lines were established in our laboratory. Both cell lines were free from mycoplasma infection. The M3 cell line was obtained from primary cultures of the BALB/c transplantable mammary adenocarcinoma, classified as estrogen receptor-negative and moderately metastatic to lung [15,18]. The F3II cell line was derived from a single cell-derived clonal subpopulation separated in vitro by limiting dilution from an M3 primary cell suspension [16].

F3II and M3 cells were seeded into 75 cm^2 plastic flasks (Corning, Corning, NY) and maintained in minimal essential medium (MEM) 410-1500 (Gibco, Grand Island, NY), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 80 μg/ml gentamicin. Cells were grown under standard tissue culture conditions at 37°C in a humidified atmosphere containing 5% CO_2. Upon reaching confluence, cells were routinely passaged using 0.25% trypsin containing 0.02% EDTA. In some instances, semiconfluent monolayers grown on glass coverslides were fixed and stained with hematoxilyn and eosin for morphological studies.

When cell suspensions were collected for experiments, harvesting was followed by washing, resuspension, and incubation in serum-free MEM for 1 hour at 37°C. Quantification of cell number was made by hemocytometer counting. In all cases, viability was >90%, as assayed by trypan blue exclusion technique. For all studies, tumor cells from passages 5–50 were used.

In Vitro Growth

Tumor cell suspensions were seeded on 9.5 cm^2 plastic Petri dishes (Corning) at a concentration of 1.5×10^5 cell/dish. Medium was changed every 2 days. Cells were trypsinized and hemocytometer counts were made daily by triplicate during 4 consecutive days. A growth curve was constructed to determine doubling times and saturation densities.

Chromosome Analysis

Log phase growing cultures were incubated in 0.15 μg/ml demecolcine (Sigma Chemical Co., St. Louis, MO) for 90 minutes. Tumor cells were then harvested, hypotonized in 0.075 M KCl, fixed in methanol/acetic acid (3:1), and stained with Giemsa, as previously described [18]. At least 100 metaphases were analyzed on both cell lines to determine chromosome frequency distribution and morphology. Normal karyotype of mouse cells is composed of 40 acrocentric chromosomes.

Fibronectin Expression

We examined immunostained patterns of fibronectin expression in semiconfluent monolayers cultured in different conditions. Tumor cells grown on glass coverslides were incubated for 48 hours in serum-free MEM or MEM plus 5% FBS, and with or without 1 μg/ml glu-plasminogen (Chromogenix, Mölndal, Sweden). Monolayers were fixed with 5% acetic acid in methanol and immunostained with goat antihuman fibronectin polyclonal antibody by streptavidin-alkaline phosphatase method [15]. Preimmune goat serum was employed as control. All reagents for immunostaining were provided by Sigma.

Preparation of Conditioned Media and Cell Lysates

Secreted and cell-associated, tumor-derived uPA activity was investigated in conditioned media and cell lysates, respectively [19]. Semiconfluent tumor cells cultured in 9.5 cm^2 plastic Petri dishes were extensively washed in phosphate-buffered saline (PBS) to eliminate serum traces. One ml of serum-free MEM was added and incubation was continued for 24 hours. Conditioned media were individually harvested and the remaining monolayers were scraped, resuspended in PBS, and lysed by sonication. Samples were centrifuged, stored at −40°C, and used only once after thawing.

Zymography and Radial Caseinolysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Laemmli [20] using 10% separating and 4% stacking gels. Gels were washed with 2.5% Triton X-100 and incubated at the surface of a plasminogen-rich casein-agarose underlay [21]. Molec-

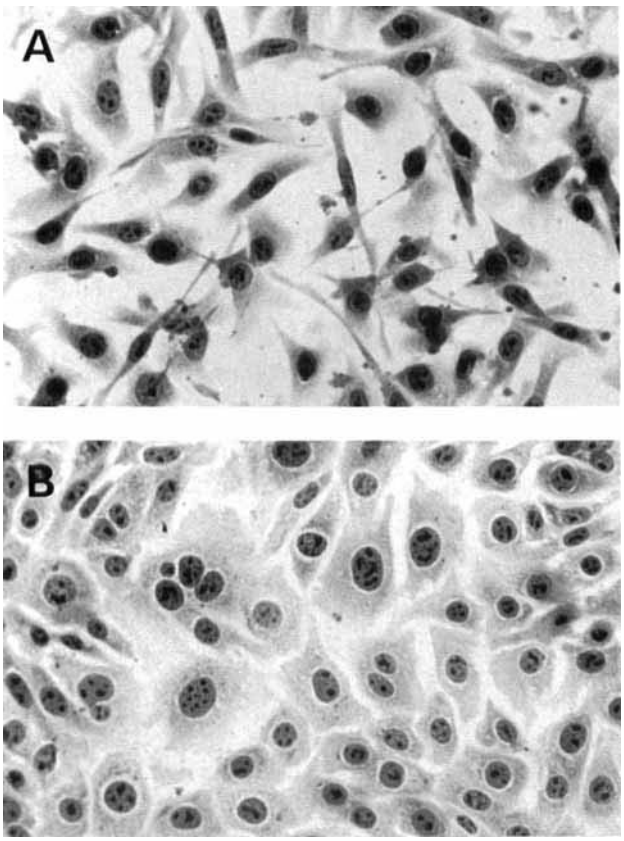

Fig. 1. Light micrographs of mammary tumor cell lines at passage 30. **A:** F3II tumor cells. **B:** M3 tumor cells. ×400 (hematoxylin and eosin staining).

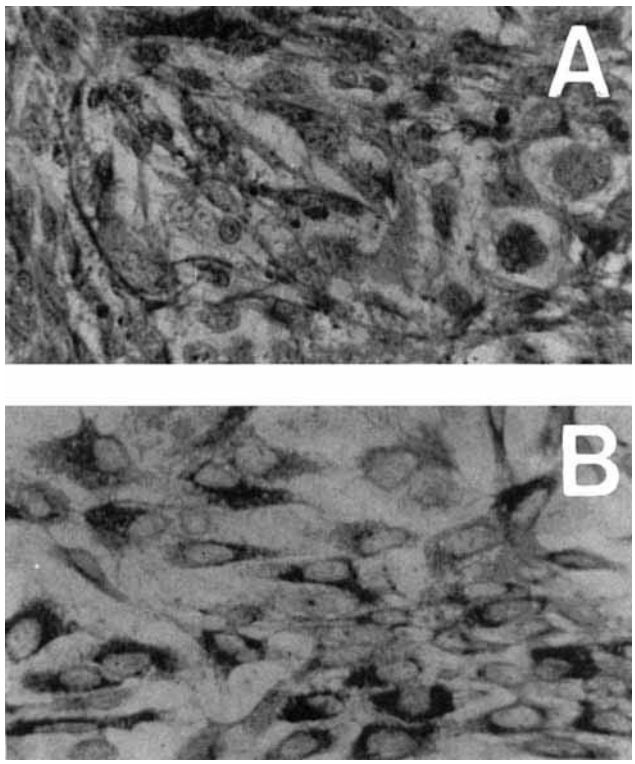

Fig. 2. Effect of serum on immunostained patterns of fibronectin expression in semiconfluent F3II monolayers cultured in the presence of plasminogen. **A:** Fibronectin was mainly expressed as an extracellular fibril network surrounding tumor cells in monolayers cultured in the presence of 5% FBS. **B:** When monolayers were cultured in serum-free MEM, most extracellular fibrils disappeared and fibronectin was found in the cytoplasm of tumor cells. ×400.

ular weights were determined using standard prestained molecular weight markers. Some samples were preincubated with anticatalytic rabbit polyclonal antibody to murine uPA (provided by Dr. G. Høyer-Hansen, Denmark). All reagents for electrophoresis were purchased from Sigma.

The method of Saksela [22] utilizing plasminogen-rich casein-agarose plates was employed to quantify uPA activity. Plasminogen-free casein-agarose gels were used to test plasminogen-independent activity [23]. Secreted and cell-associated uPA activities were referenced to the original number of tumor cells.

Cell-Surface uPA Activity

Semiconfluent tumor cells were cultured in the conditions described above and washed in PBS. One ml of serum-free MEM containing 1 mg/ml of bovine serum albumin was added and incubation was continued for 24 hours. Receptor bound uPA was then removed as described by Jankum et al. [24]. Briefly, monolayers were washed with PBS and treated 5 minutes at room temperature with 0.75 ml of 0.05 M glycine-HCl buffer containing 0.1 M NaCl (pH 3.0). The acid eluate was quickly neutralized with 0.25 ml of 0.5 M Tris-HCl (pH 7.8) and stored at −40°C until assayed for uPA activity by zymography and radial caseinolysis. The remaining monolayers were lysed by sonication and analyzed as described above.

In Vivo Studies

Female and male BALB/c inbred mice with an average age of 14 weeks and a weight of 25–30 g were used.

To evaluate growth behavior, local invasiveness, and spontaneous metastatic ability, animals received 2×10^5 tumor cells from different in vitro passages, resuspended in 0.2 ml of serum-free MEM. For each experiment, at least eight mice were given inoculations in the subcutis of the right flank. The mean of the two largest perpendicular diameters was recorded twice a week to evaluate local tumor growth. At different times postinoculation, animals were sacrificed by cervical dislocation and necropsied. To study histopathologic features and local invasion, tumors were fixed in 10% formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin or Mallory's aniline blue collagen stain. To investigate the presence of spontaneous metastases, lungs were re-

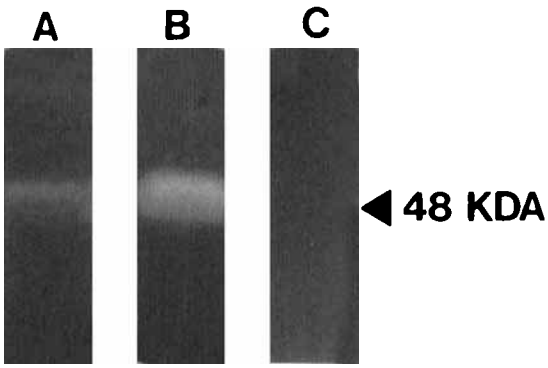

Fig. 3. Zymographic analysis of plasminogen activators produced in vitro by F3II cells. Gels were incubated at the surface of a plasminogen-rich casein-agarose underlay. proteolytic enzymes were detected as transparent bands on the background of Amido black-stained underlays. Lane **A:** Cell lysate. Lane **B:** Conditioned medium. Lane **C:** Conditioned medium preincubated with 10 μg/ml anticatalytic rabbit polyclonal antibody to murine uPA.

moved and fixed in Bouin's solution. The number of surface lung nodules was determined under a dissecting microscope. Some lungs were used for histological examination. Liver, kidney, and spleen also were examined for the presence of metastatic nodules.

To examine the ability of tumor cells to produce experimental lung metastases, tumor cells at a concentration of 2×10^5 cells/0.3 ml MEM were injected into the lateral tail vein of unanesthetized mice. Animals were monitored daily and sacrificed 3 weeks later by cervical dislocation. Lungs were studied as described above.

In another experiment, tumor cells were injected into the peritoneal cavity, at a concentration of 5×10^5 cells/0.3 ml MEM/mouse. Animals were monitored for the formation of ascitis, sacrificed at day 25 and necropsied.

Tumor-Induced Angiogenesis

Each mouse was given a suspension of tumor cells intradermally (1.5×10^5 cells in 0.1 ml of MEM). After 5 days, the mice were killed and the skin was examined under a dissecting microscope. Quantification of vascularization was done by measuring the vessel density, as described [25].

Electron Microscopy and Immunocytochemistry

Tissues for ultrastructural and immunocytochemical analysis were obtained from subcutaneous tumors, 25 days after inoculation of tumor cells. For transmission electron microscopy, tumor samples were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetraoxide in phosphate buffer 0.1 M, and embedded in Epon. Ultramicrotome sections, contrasted with uranile acetate and lead citrate, were prepared. Representative areas, as identified in toluidine blue-stain 1 μm sections, were examined and photographed in a Zeiss EM 109 at 80 KV. For immunoperoxidase staining 5-μm sections were cut, mounted, deparaffinized, rehydrated, and treated with 0.5% H_2O_2 in methanol to block endogenous peroxidase activity. Primary antibodies to cytokeratin or vimentin (Dako Corporation, Carpinteria, CA) were applied, the avidin-biotin-peroxidase complex was added and diaminobenzidine was used as chromogen substrate [26].

RESULTS

In Vitro Features

The F3II cell line has been in culture for >50 passages. It has grown as elongated cells adherent to the bottom of the flask since it was established (Fig. 1A). Tumor cell morphology was not substantially modified either during in vitro passages or after freezing and thawing (passages 9 and 20). F3II monolayers were mostly composed of fusiform cells, homogeneous in their cytoplasmic and nuclear size and shape. Cells frequently grew in a whorllike pattern. The parental, nonselected M3 cell line was composed of epithelioid polyhedric cells, heterogenous in their size and shape (Fig. 1B). Occasionally, some giant and multinucleated cells were observed, as reported previously [15,16].

Cell Growth Properties

When cultured in MEM containing 5% FBS, the growth rates of F3II and M3 cells were similar, with population doubling times of 17.7 and 19.2 hours, and saturation densities of 1.8×10^5 and 2.2×10^5 cells/cm^2, respectively, at passage 18. Similar results were obtained at passage 35.

TABLE I. Levels of Cell-associated and Secreted Urokinase-type Plasminogen Activator (uPA) Activity of F3II and M3 Cell Cultures†

Cell line	Secreted (IU/24 h/10^6 cells)	Cell-associated		
		Total (mIU/10^6 cells)	Receptor-bound (%)	Cytoplasmic (%)
M3	17.6 ± 3.0	885 ± 124	15 ± 13	85 ± 13
F3II	5.4 ± 0.9*	311 ± 69**	68 ± 5**	32 ± 5**

†Data from passage 25 are shown. Values represent means ± SD of triplicate determinations, and are representative of five sets of experiments using monolayers from different in vitro passages.
*$P < 0.001$ and **$P < 0.01$, Student's t-test.

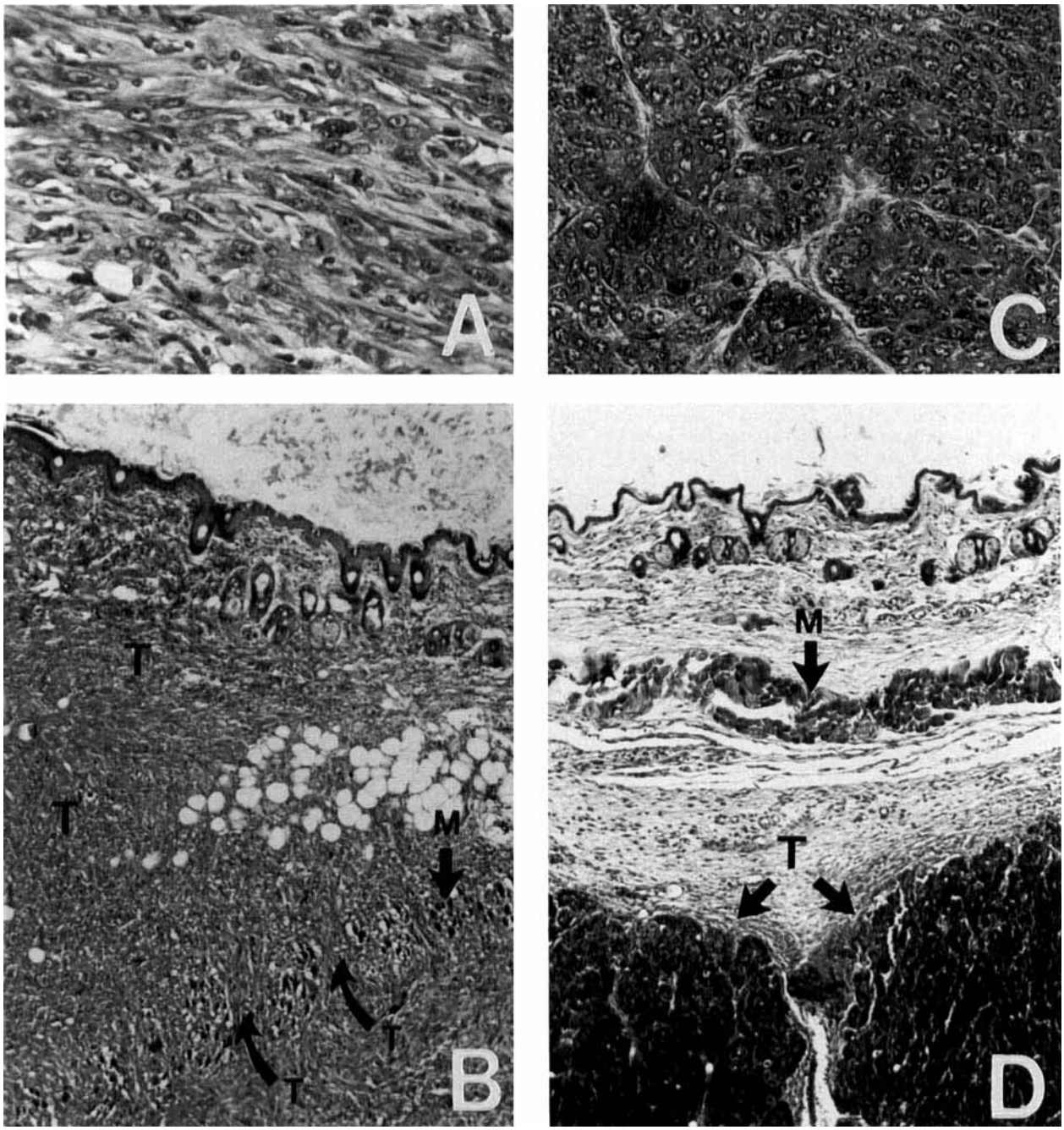

Fig. 4. Light micrographs of paraffin sections from subcutaneous tumors produced by F3II and M3 cells. **A:** Sarcomatoid aspect of F3II tumor cells. ×400. **B:** Invasive properties of F3II sarcomatoid carcinoma tumors. High degree of invasiveness of muscle and adipose layers of the subcutis and dermis. ×100. **C:** M3 cells produced moderately-to-poorly differentiated adenocarcinomas. ×400. **D:** The absence of local invasion in M3 tumors. Tumors grew without signs of active invasion of muscle layer or dermis. ×100. Muscle layer (M) and tumor cells (T) are indicated (Mallory's aniline blue collagen staining).

Chromosome Number

F3II tumor cells proved to be nearly triploid, whereas M3 cells were in the tetraploid range. Highly significant differences ($P < 0.01$, Student's t-test) were found between the mean chromosome number of F3II cells (56.5 ± 1.6) with respect to M3 cells (76.0 ± 2.2), at passage 27. Modal chromosome numbers were 57 for F3II and 84 for M3. In both cases, most chromosomes (up to 98%) were acrocentric. Similar results were obtained at passage 41.

Expression of Fibronectin and uPA

Fibronectin was mainly expressed as an extracellular fibril network surrounding F3II cells in monolayers cultured in the presence of 5% FBS (Fig. 2A). When cells were cultured in serum-free MEM, most extracellular fibrils disappeared and fibronectin was found in the cytoplasm as coarse granular staining material (Fig. 2B). In the absence of serum protease inhibitors, tumor proteolytic activity was responsible for the degradation of extracellular fibronectin. The degradative activity was more evident in the presence of plasminogen, indicating the production of plasminogen activators by tumor cells. Similar results were obtained with M3 monolayers (data not shown).

Zymographic assay of cell lysates and conditioned media from both F3II and M3 cultures showed that plasminogen activator activity corresponded to a main band of 48 kDa, confirming previous results [16,19]. This protease activity was completely abolished by preincubation with anticatalytic antibodies to murine uPA (Fig. 3). Plasminogen-independent activity was never detected in the present assay conditions.

The data in Table 1 show that F3II exhibited lower levels of secreted and cell-associated uPA activities than M3 monolayers. However, F3II cells exhibited an important proportion of receptor-bound (acid-labile) uPA activity. In contrast, M3 cells showed low levels of receptor-bound uPA and a high percentage of cytoplasmic (acid-resistant) uPA activity.

In Vivo Behavior

Subcutaneous tumors originated from F3II cells were diagnosed as invasive sarcomatoid carcinomas, since they were mainly composed of spindle cells (Fig. 4A). The cell line fully retained its histopathologic character after in vitro passages. F3II cells grew by invading the muscular and adipose layers of the subcutis (Fig. 4B). At 25 days after inoculation, tumor cells also invaded the dermis and the dermal papillae in most animals, causing necrosis in the epidermal layer and visible ulceration on top of the tumors. Histologic examination of the lungs from tumor-bearing mice revealed metastatic nodules recognized as poorly differentiated adenocarcinoma. In several instances, metastatic nodules presented sarcomatoid aspect. Some nodules were perivascular, suggesting a hematogenous spread. On the other side, M3 tumors were classified as moderately-to-poorly differentiated adenocarcinomas (Fig. 4C). M3 cells grew by filling the subcutis and without signs of active invasion of subcutaneous muscle layer or dermis (Fig. 4D).

Tumorigenicity and metastatic ability of F3II and M3 cells are summarized in Table II. No differences were observed in the latency period between the new tumor variant F3II and the parental M3 line. However, F3II showed a significantly lower tumor growth rate and higher mean survival time. The incidence of spontaneous lung metastases were higher in F3II- (90%–100%) than in M3-bearing animals (40–60%). Interestingly, the higher incidence of spontaneous metastases was not merely an epiphenomenon of the longer survival time of F3II hosts. On the contrary, there were no significant differences in the number or size of experimental lung metastases between the two lines. Extrapulmonary tumor colonies were not found in any case. No important differences were observable between the growth on female or male BALB/c mice for both cell lines. F3II produced significantly higher angiogenic responses than M3 tumor cells (3.4 ± 0.6 vs. 2.3 ± 0.4 vessels/mm^2; $P < 0.01$, Mann-Whitney U test).

In addition to forming a suspension of tumor cells when injected in the peritoneal cavity, F3II cells grew as invasive solid tumors in the bowel wall. Ascites fluids typically became bloody by day 20; blood loss into the peritoneal cavity contributed to animal death. In contrast, M3 cells commonly formed noninvasive solid tumor implants in the mesentery and in the parietal peritoneum.

Ultrastructural and Immunocytochemical Studies

Electron microscope analysis of F3II subcutaneous tumor cells demonstrated ultrastructural characteristics typical of carcinomas. These features included surface microvilli, numerous ribosomes and rudimentary intercellular junctions (Fig. 5). However, desmosomes were not common and were incompletely developed; tonofilaments were not apparent. Nuclei were generally large and irregularly shaped and sometimes contained multiple nucleoli or areas of chromatin condensation. A moderate number of mitochondria and a defined rough endoplasmic reticulum were observed. Some tumor cells contained dilated cisterns with few viruslike A particles (Fig. 6). Most ultrastructural characteristics were similar to those observed for parental M3 adenocarcinoma tumors [15]. Immunocytochemical staining of F3II cells exhibited significant expression of cytokeratin and absence of vimentin, indicating an epithelial origin as demonstrated for M3 [26].

DISCUSSION

It has been established that despite the monoclonal origin of most tumors, they are very heterogeneous in nature [1–3]. Within a tumor, several tumor cell clones

TABLE II. Tumorigenic and Metastatic Ability of F3II and M3 Mouse Mammary Tumor Cell Lines†

	F3II	M3
Latency, days [median (range)]	6 (5–12)	7 (6–12)
Tumor growth rate, mm/day [mean ± SD]	0.44 ± 0.11*	0.87 ± 0.17
Tumor size at day 25, mean diameter in mm [mean ± SD]	9.4 ± 1.5*	17.6 ± 3.4
Histopathology	Sarcomatoid carcinoma	Adenocarcinoma
Local tumor invasion	Present	Absent
Spontaneous lung metastases		
Incidence [percentage]	100**	50
Number of surface nodules [median (range)]	4 (2–10)***	2 (0–12)
Survival, days [mean ± SD]	69 ± 7****	42 ± 3
Cause of death	Lung metastases	Subcutaneous tumor burden
Experimental lung metastases		
Number of surface nodules [median (range)]	17 (5–26)***	10 (1–28)

† Data from passage 26 are shown (passage 29 for experimental lung metastases). Each group consisted of at least 8 male mice. Values are representative of different experiments using cells from in vitro passages 5–50. No sex-dependent growth or metastatic properties were observed for both cell lines. * $P < 0.01$ and **** $P < 0.001$, Student's t-test; ** $P < 0.01$, Chi-square test; *** nonsignificant, Mann-Whitney U test.

coexist exhibiting a different proliferation rate, morphology, and metastatic potential. We must, however, bear in mind that whatever heterogeneous behavior metastatic cells may display, they must always exhibit the necessary requirements that comprise the malignant phenotype. The identification of such requirements depends on the availability of animal tumor systems.

It has been shown that tumor cell subpopulations with a different metastatic potential could be isolated from a tumor line [1,11,27]. These cells seemed to have special properties that enable them to complete the highly inefficient metastatic process, where <0.1% of the tumor cells released from the primary tumor may survive arrest in target organs and even fewer may produce a metastatic focus [14].

In the present study we characterized the new F3II mammary tumor cell line, previously established in vitro from a single-cell-derived clonal subpopulation of the mouse mammary adenocarcinoma M3 [16]. The subcutaneous tumors induced by F3II cells were classified as poorly differentiated, spindle-cell carcinomas ("carcinosarcomas") with a prominent local invasiveness and a higher spontaneous metastatic ability than the M3 parental tumor.

The origins of the concepts pertaining to the histogenesis of carcinosarcomas can be traced to theories that were advanced >100 years ago [28,29]. In 1906, Ehrlich and Apolant [30] found that after several successive inoculations of an adenocarcinoma in mice, it began to adopt a spindle-cell, sarcomalike microscopic appearance. Since that time, debate has continued over the clonality of these neoplasms and their lineages of differentiation. Our experimental data may indicate a monoclonal nature for mamary carcinosarcoma in mice and support that "sarcomatoid carcinoma" is more apt designation for this tumor entity, as suggested by others [29,31]. It is conceivable that many human breast tumors behave similarly. In addition, most published analyses of the behavior of human mammary sarcomatoid carcinomas have shown a less favorable clinical outcome than other types of breast carcinoma [31,32].

The enhanced aggressiveness of F3II tumor cells correlated with some in vivo and in vitro features. F3II cells produced highly angiogenic responses. In this regard, progressive tumor growth followed by metastasis appears to be associated with neovascularization [25]. Although F3II cells produced lower levels of the degradative enzyme uPA, they showed a higher proportion of cell-surface uPA than M3 cells; high-affinity cell-surface receptors focus uPA activity close to the plasma membrane, where it is more useful to invasive cells [8,9]. F3II proved to be triploid, whereas M3 monolayers were in the tetraploid range. In accordance, aneuploidy is usually associated with tumor progression [33].

The acquisition of the metastatic phenotype may depend on the inherent phenotypic instabilities of the tumor cells that compose primary neoplasms [34]. However, the pressures from the microenvironment may be able to select transient tumor cell subpopulations that could lead, in time, to the generation of metastasis.

CONCLUSIONS

We characterized a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a

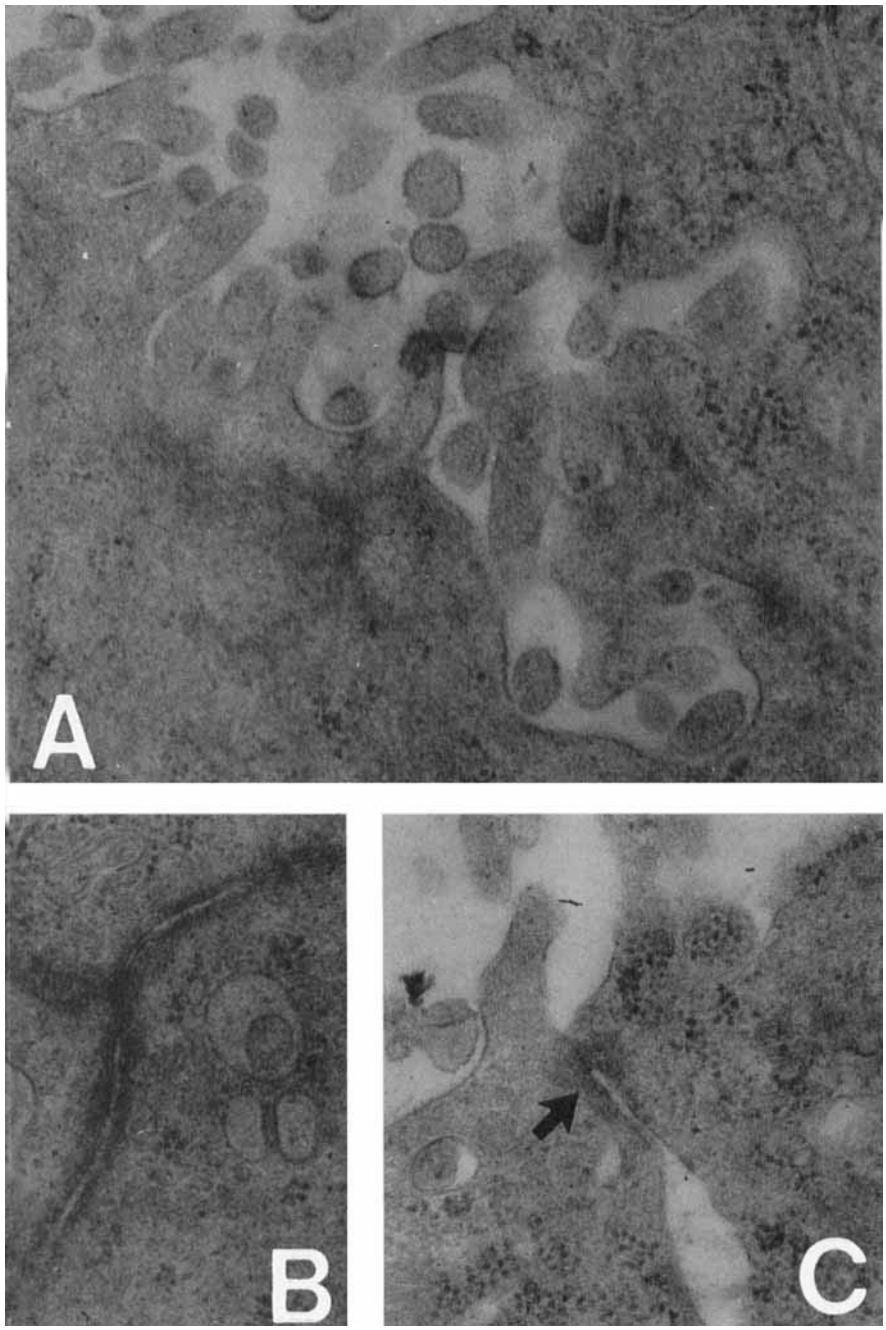

Fig. 5. Ultrastructural features of F3II cells. **A:** Glandular lumen with short microvilli. **B:** Rudimentary intercellular junction. **C:** Primitive cell junction near a glandular lumen (arrow). ×20,000.

BALB/c mouse adenocarcinoma. The data presented in this study indicate that the new line is less differentiated and more aggressive than the original tumor variant. Taking a wider perspective, these findings support the notion that breast carcinomas are heterogeneous neoplasms and contain subpopulations of tumor cells with diverse biologic behavior.

The new F3II mouse mammary sarcomatoid carcinoma line is a suitable model system to examine the role of degradative enzymes that facilitate tumor cell invasion. Furthermore, this tumor model may be useful to test antiinvasive, antiangiogenic, and antimetastatic agents.

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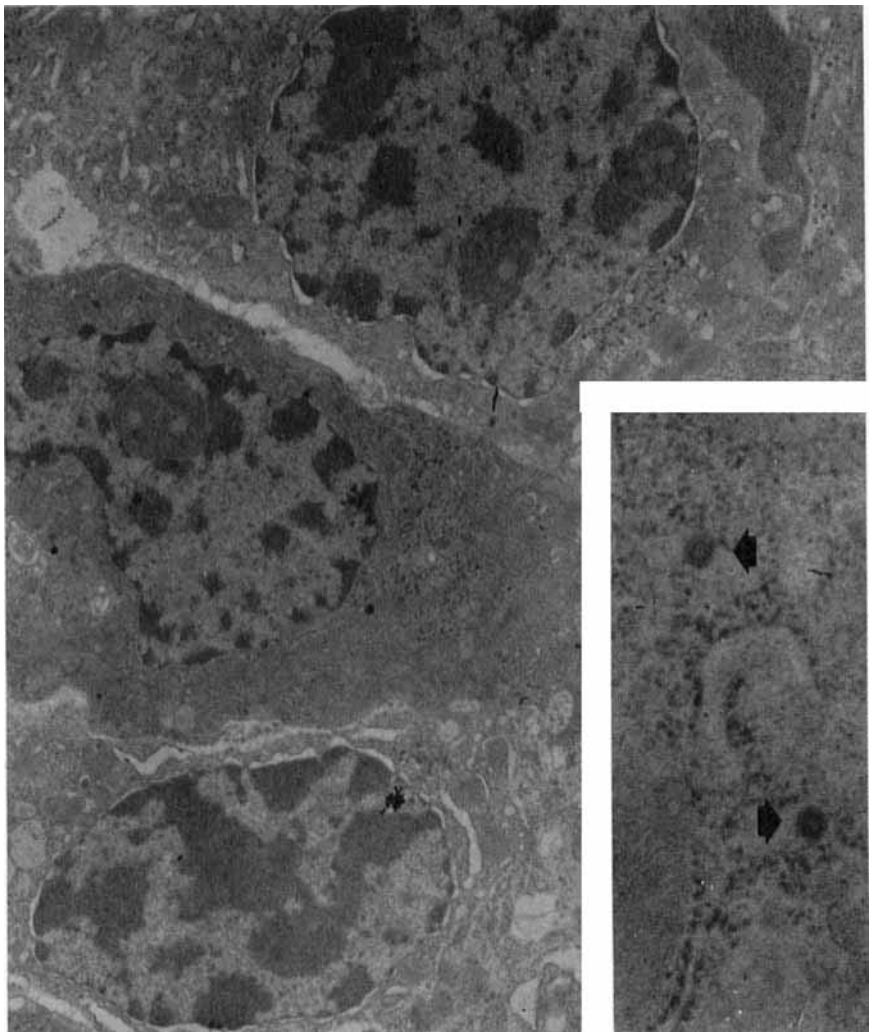

Fig. 6. Ultrastructure of F3II subcutaneous tumor cells. ×3,000. Inset shows some dilated cisterns containing viruslike A-type particles. ×20,000.

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