Tumorigenicity and Metastasis of Human Breast Carcinoma Cell Lines in Nude Mice

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ABSTRACT

There are few reports describing experimental models of the growth and metastasis of human breast carcinomas. This article discusses the tumorigenic and metastatic properties of two estrogen receptor-negative breast carcinomas injected into nude mice.

Tumor growth in the mammary fat pad (m.f.p.) and the subcutis was compared in female nude mice. The injection of 10^6 viable cells of two human breast carcinoma cell lines (MDA-MB-231 and MDA-MB-435) gave a 100% tumor take rate in the m.f.p., whereas only 40% of the s.c. injections produced tumors and these occurred several weeks after the appearance of the m.f.p. tumors. Thus, the m.f.p. of nude mice is a favorable site for the growth of human breast carcinomas. MDA-MB-435 tumors produced distant metastases in 80% to 100% of recipients. The most common sites for metastases were the lymph nodes and lungs, with a lower incidence of metastases in muscle (chest wall and thigh), heart, and brain. New variant cell lines were isolated from metastases in the lungs, brain, and heart. All the cell lines were tumorigenic in the m.f.p., and the lung- and heart-derived metastasis lines produced slightly more lung metastases than the original cell line. However, the brain metastasis variant produced significantly fewer lung metastases. Intra-venous inoculation of the spontaneous metastasis-derived cell lines produced few lung colonies. Only cell variants isolated from experimental lung metastases showed enhanced lung colonization potential when re-injected i.v.

Our results suggest that the estrogen receptor-negative MDA-MB-435 cell line injected in the m.f.p. of nude mice could be a valuable tool for analysis of the cellular and molecular basis of the metastasis of advanced breast cancer.

INTRODUCTION

Since the initial observation made by Rygaard and Povlsen (1) that a human colon adenocarcinoma grew progressively in nude mice, this athymic mutant strain of mouse has been frequently used in human cancer research. Numerous studies have reported that, although not all human tumors can be successfully xenografted, the histology and biochemical properties of the tumors that do grow in nude mice closely resemble those of the original tumor specimens (2). A major use of nude mice is to prescreen chemotherapeutic agents that act against human tumors proliferating in vivo, rather than using in vitro assays or murine tumor models (3, 4).

The nude mouse also provides a means to study the biology of human tumor metastasis (5). Whereas some early reports noted that the incidence of metastasis of xenografted human tumors in the nude mouse was low (2, 6), more recent studies reveal that the metastatic phenotype can be expressed in this model system. Variables affecting whether metastasis occurs include the health and housing conditions of the mice (7, 8) and the routes of tumor cell inoculation employed (5, 9) in addition to the intrinsic properties of the tumors under investigation (10, 11). Many human tumors can proliferate when injected s.c. into nude mice, but metastasis from this site is rare (2, 6).

However, the implantation of human tumor cells into anatomically appropriate (orthotopic) sites, rather than into ectopic sites, has been shown to allow the expression of the metastatic phenotype, e.g., colon carcinoma cells into the cecum wall (11, 12), renal cell carcinoma cells into the renal subcapsule (13), and bladder carcinoma cells into the bladder (14). In addition, the delivery of cells directly to a target organ by injection into the blood stream, into the carotid artery, or into the spleen to produce metastases in lung (15), brain (16), and liver (10), respectively, is a method used to study the metastatic colonization potential of human tumors.

Human breast carcinomas have a low tumor take rate in nude mice (2), and there are few reports of metastasis of these xenografted tumors. ER*-positive MCF-7 cells, growing in the m.f.p. of female nude mice supplemented with estrogen, can metastasize to the lungs and lymph nodes (17). Ozzello and Sordat (18) reported two transplantable human breast carcinomas that grew rapidly in nude mice and also formed metastases in the lungs and lymph nodes. In addition, one ER-negative cell line, MDA-MB-231, that is commonly used for in vitro studies has been reported to form lung metastases after i.v. injection (19). In this article we describe the tumorigenic and metastatic behavior of another ER-negative cell line, MDA-MB-435, which has not been previously reported as tumorigenic in nude mice. Our results show that implantation and growth of this cell line in an orthotopic site, the m.f.p., produce metastases in several different organs in the mice and thus provide a model for the metastasis of a highly aggressive, human breast carcinoma.

MATERIALS AND METHODS

Animals. Female athymic nude mice (NCr-nu/nu), 6 to 8 weeks old, were obtained from Simonsen Laboratories (Gilroy, CA). The animals were housed under specific pathogen-free conditions.

Cell Lines. MDA-MB-231 and MDA-MB-435 were generous gifts from Dr. R. Cailleau, Department of Medicine, M. D. Anderson Cancer Center (Houston, TX). Both are ER-negative cell lines isolated from the pleural effusions of patients with breast carcinoma (20, 21). HT-29 is a human colon carcinoma cell line (22), A375 is a human melanoma cell line (23), and SN12C was established from a renal cell carcinoma (13). All the cell lines were confirmed as human by karyotypic or isoenzyme analysis.

All cell lines were examined for and found to be free of reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, k-virus, Theiler's virus, Sendai virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Walkersville, MD) and Mycoplasma (tested using a Gen-Probe Mycoplasma detection kit; Gen-Probe, Inc., San Diego, CA).

Cell Culture. The cells were maintained in monolayer culture in Eagle's minimum essential medium supplemented with 5% fetal bovine

Received 7/7/89; revised 10/5/89; accepted 10/24/89.

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1 Supported in part by a grant from Triton Biosciences, Inc.
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The abbreviations used are: ER, estrogen receptor; HBSS, Hanks' balanced salt solution; m.f.p., mammary fat pad.
serum, sodium pyruvate, L-glutamine (2 mM), nonessential amino acids, and 2x vitamin solution (GIBCO, Grand Island, NY). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Tumor cells were harvested for passage or inoculation by washing the monolayer with HBSS, followed by a brief incubation in 0.25% trypsin and 0.02% EDTA. The tissue culture flask was tapped to dislodge the cells, which were then resuspended in minimum essential medium supplemented with 5% fetal bovine serum. Tumor cells intended for inoculation into mice were washed by centrifugation and resuspended in HBSS. Cell number and viability were determined by staining a small volume of cell suspension with 0.2% trypan blue saline solution and examining the cells in a hemocytometer.

Tumor Cell Inoculation. In preparation for inoculation into the m.f.p., the mice were anesthetized with Metofane (Pitman Moore Inc., Washington, NJ) and a 5-mm incision was made in the skin over the lateral thorax. The m.f.p. was exposed, and a volume of 0.1 ml of cell inoculum was injected into the tissue through a 27-gauge needle. By exposing the fat pad, we were able to ensure that the cells were injected into the tissue and not into the s.c. space. Tumor cells were also injected s.c. (lateral flank) or i.v. (lateral tail vein) in separate experimental groups. The growth of m.f.p. and s.c. tumors was monitored by weekly examination, and growth rates were determined from caliper measurements of two diameters. In some experiments the tumors were excised at a mean diameter of 1.5 cm; in other experiments, they were excised at variable times after cell inoculation. The mice were anesthetized, the tumors were removed, and the skin incisions were closed with wound clips. Experiments were terminated 4 weeks after tumor excision or when the mice were moribund. For the i.v. injection experiments, the mice were killed 12 weeks after injection or when moribund.

Isolation of Metastasis-derived Variants. At autopsy, metastases were dissected from different organs (lungs, brain, heart, and lymph nodes), minced finely, and plated in tissue culture flasks in culture medium. The resulting cell lines were tested for contamination with mouse cells by isoenzyme analysis (Authentikit; Innovative Chemistry, Marshfield, MA). All of the metastasis-derived lines were of human origin, with no mouse cell contamination.

Growth of Human Breast Carcinoma Cells on Cytodex Beads. Cytodex-3 beads (Pharmacia) were rehydrated, following the manufacturer’s instructions, and sterilized. Beads (2 x 10⁶/ml) and cells of the two breast carcinoma cell lines (2 x 10⁵/ml) were plated in 10 ml of culture medium in 100-mm Petri dishes (i.e., not tissue culture-treated dishes; the tumor cells adhered to the beads and not to the dishes). After 3 to 5 days, when the beads were covered with cells, a sample was taken and trypsinized to estimate the numbers of cells per bead. For MDA-MB-231, this number was 75 cells/bead and for MDA-MB-435, 28 cells/bead. Beads coated with cells were washed with HBSS and injected i.v. into groups of nude mice, approximately 5 x 10⁶ beads in 0.2 ml of HBSS/mouse. Eight to 10 weeks after injection, the mice were killed and the lumps were examined for tumor colonies.

RESULTS

Site of Tumor Cell Injection: s.c. versus m.f.p.. To test whether the site of injection influenced the growth of human breast carcinoma cell lines in nude mice, we injected cells s.c. and into the m.f.p. of mice and compared the tumor take and subsequent growth rates. For two human breast carcinoma cell lines, MDA-MB-231 and MDA-MB-435, three inoculum doses were used. At the two higher doses (10⁶ and 10⁵ cells), tumors developed in all mice at both sites of injection. However, at the lowest cell dose (10⁵ cells), tumors grew in 2 of 5 mice injected s.c., whereas 5 of 5 mice injected into the m.f.p. developed tumors; this result was the same for both cell lines in two repeated experiments. Fig. 1 shows that the latent interval for appearance of MDA-MB-435 m.f.p. tumors was less than that for s.c. tumors, following injection of 10⁵ cells; the growth curves for MDA-MB-231 tumors were similar to those of MDA-MB-435 shown here. At the two higher cell doses, tumors appeared in both sites after similar intervals.

To determine whether the trophic effect of the m.f.p. was specific for breast carcinoma cells, we tested the growth of three different human tumor cell lines injected s.c. or into the m.f.p. For a melanoma (A375), a colon carcinoma (HT-29), and a renal cell carcinoma (SN12C), there was no difference in tumor take or growth rate following injection into the m.f.p., as compared with growth of the same number of cells (10⁵ cells) injected s.c. (Fig. 1). A375 and SN12C cells produced tumors in all mice given injections in either site (5 of 5), and HT-29 cells produced tumors in 4 of 5 mice given injections s.c. or into the m.f.p. The experiment was performed twice, with identical results. Based on the results from these experiments with MDA-MB-231 and MDA-MB-435, we adopted the m.f.p. as the site of injection in developing the model of human breast carcinoma metastasis in nude mice.

The histology of the MDA-MB-435 cell line is that of a poorly differentiated adenocarcinoma (Figs. 2 and 3). When
Thirty female nude mice were given injections of $5 \times 10^6$ MDA-MB-435 cells into the m.f.p. At 4, 8, and 12 weeks after injection, the tumors were excised from a group of 10 mice. At 16 weeks after the initial injection, all the mice were killed and examined for metastasis.

Metastasis from Tumors Growing in the Mammary Fat Pad. Both MDA-MB-231 and MDA-MB-435 cell lines formed progressively growing tumors following injection of cells ($10^5$ to $10^6$ cells) into the m.f.p. Of 7 mice with MDA-MB-231 tumors that were killed 20 weeks after tumor cell injection, only 1 was found to have macroscopic lung metastases. In contrast, in repeat experiments, 80 to 100% of mice with MDA-MB-435 tumors developed metastases, primarily in the lymph nodes and lungs. Metastases also were found at a lower frequency in the brain, heart, adrenal glands, and muscle (chest wall and thigh) (Tables 1 and 2). The histological appearance of a brain metastasis is shown in Fig. 4. The lesions are of a poorly differentiated carcinoma, and bizarre mitotic figures are not uncommon. Subcutaneous MDA-MB-435 tumors produced fewer lung metastases, in 20 to 40% of tumor-bearing mice.

To determine the time course of metastasis, MDA-MB-435 m.f.p. tumors were excised at 4, 8, and 12 weeks after injection. At 16 weeks, all mice were killed and the numbers and locations of metastases were recorded. Table 1 shows that by 4 weeks tumor cells had disseminated in 3 of 10 mice and by 8 weeks, at which time the mean weight of the m.f.p. tumors was 0.8 g, distant metastases were already established in 70% of recipients.

All of the mice with tumors present for 12 weeks developed distant metastases. A similar analysis of the metastasis from s.c. tumors of weights comparable to those shown in Table 1 was not performed, because most s.c. tumors did not reach the largest weights; as noted above, the s.c. tumors became necrotic at smaller sizes than the m.f.p. tumors.

The results from this experiment show that metastatic cells disseminate from the m.f.p. tumors at a relatively early stage (in some animals before 4 weeks; average tumor weight, 130 mg) but that the frequency of metastasis is associated with tumor burden. In a related study, a smaller inoculum of MDA-MB-435 cells was injected into the m.f.p., and tumors excised 8 to 10 weeks after injection weighed 0.4 to 0.55 g (i.e., lower weights than the group reported in Table 2). Metastases were found in a smaller percentage (40%) of these animals killed at 16 weeks after the initial injection.

Metastatic Ability of Metastasis-derived Variants of MDA-MB-435. Variants of the MDA-MB-435 cell line were established from metastases in nude mice that had m.f.p. tumors of the original cell line. 435-Lung1 and 435-Lung2 were established from lung metastases, with the latter originating from a metastasis of the 435-Lung1 cell line. 435-Br1 was isolated from a brain metastasis of MDA-MB-435.

When injected into the m.f.p. of nude mice, all of the metastasis-derived cell lines grew at similar rates and were not differ-

![Fig. 3. MDA-MB-435 s.c. tumor in the lateral flank of a nude mouse. × 100.](image)

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<th>Table 2 Metastasis of MDA-MB-435 and selected variants from mammary fat pad tumors to the lungs of nude mice</th>
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![Fig. 4. MDA-MB-435 metastasis in the brain of a nude mouse. × 100.](image)
ent from the parental cell line. The two lung metastasis-derived lines, from one or two cycles of growth in the m.f.p. and metastasis to the lungs, both produced high numbers of lung metastases and at a slightly earlier autopsy time, as compared with the MDA-MB-435 parental cell line (15 versus 16–20 weeks) (Table 2). However, the only significantly different lung metastasis formation resulted from injecting the brain metastasis-derived cell line into the m.f.p. Only 3 of 10 mice developed macroscopic lung metastases; in two repeat experiments, a similarly low incidence of lung metastasis from 435-B1 cell colony tumors was found (data not shown). Lymph node metastases were found in 4 of the 10 recipient mice. None, however, showed obvious symptoms of brain metastasis. Each brain was sectioned on several levels to screen for metastases, and small foci of carcinoma cells were detected in the brains of 2 mice from the 435-B1 group.

The ability of the MDA-MB-435 cells and the metastasis-derived variants to form lung colonies was assessed by injecting 10^6 cells i.v. via the tail vein. None of the cell lines showed marked ability to colonize the lungs, producing few lung colonies in only 2 or 3 of 10 mice injected in each group.

Experimental Lung Metastasis by Human Breast Carcinoma Cells Injected as Single Cells or Attached to Cytodex Beads. The rationale for injecting breast carcinoma cells cultured on the Cytodex beads was to establish whether the cells could proliferate in the lungs once they were arrested by physical trapping in the large beads (100-μm diameter). The results in Table 3 show that MDA-MB-231 cells delivered to the lungs of nude mice on the beads proliferated and formed macroscopic lesions in 7 of 7 mice injected. The estimated dose of MDA-MB-231 cells injected on the beads was 4 × 10^6 cells; double this cell dose injected as a mononuclear suspension failed to form any experimental metastases within 12 weeks of the injection. The cell line established from the lung tumor in mice given injections of MDA-MB-231 cells grown on Cytodex beads (231-Bead1) also did not form lung metastases after injection as single cells.

The results with the MDA-MB-435 cell line were essentially similar (Table 3), with an increase in the incidence and number of experimental lung metastases when the cells were injected on Cytodex beads (estimated dose of 1.4 × 10^6 cells) rather than injected as a mononuclear suspension. However, the 435-Bead1 variant cell line (from lung tumors of mice given injections of MDA-MB-435 cells grown on Cytodex beads) did show an increased lung colonization potential when injected as single cells. A cell line established from the few lung colonies of MDA-MB-435 injected i.v., designated MDA-435-PM1, also had a higher lung colonization potential than the original cell line. Thus, for the MDA-MB-435 cell line, growth in the lungs of nude mice appeared to select for cells with enhanced lung-colonizing properties; these cells were possibly only a small subset of the parent cell line, indicated by the result that 1 of the 10 mice given injections of single cells developed lung colonies (Table 3).

Essentially, the experiments with the Cytodex beads showed that the human breast carcinoma cells were capable of growth in the nude mouse lungs and that the poor lung colonization after i.v. injection of 10^6 single cells was possibly due to few cells arresting in the lungs, rather than an inability to grow in this organ.

**DISCUSSION**

Use of athymic nude mice has allowed many and diverse studies of human tumor biology (1–3), including in relatively recent years the analysis of metastatic properties (6, 9–15). In this report, we describe a new experimental model of the metastasis of an ER-negative human breast carcinoma cell line following implantation and growth in the m.f.p. of nude mice.

The initial question was whether the route of injection of breast carcinoma cells in the nude mice would influence tumorigenicity and metastasis, as seen for different types of human tumors (11–14). The m.f.p. is a more favorable site for the growth of mouse mammary tumors, compared with the subcutis (24), and also for the development of metastases, with a higher frequency of metastasis from the m.f.p. tumors (25). It has been shown previously that both cleared and intact m.f.p. in nude mice support the growth of human neoplastic breast tissue (17, 26). In this study of five human tumor cell lines injected i.s. and into the m.f.p., only the two breast carcinoma cell lines showed enhanced growth in the m.f.p. at the low inoculum dose. Preferential growth in the m.f.p. site for all tumors would suggest a general effect, resulting, for example, from a good blood supply in the glandular tissue as compared with the subcutis. The trophic effect on breast carcinomas and not the other tumors may suggest specific tumor-host cell interactions that create a favorable environment for neoplastic breast cells and allow growth of tumors at a lower inoculum dose than is required in the subcutis. Conversely, the s.c. site is comparatively restrictive to the growth of breast carcinoma cells and, thus, the threshold tumorigenic dose is higher, as compared with that of the m.f.p. site. At higher inoculum doses, possibly above the threshold for s.c. growth, differences in tumor take and latent interval in the two sites were not found. The results of our study recommend the m.f.p. site for the inoculation of freshly isolated human breast carcinomas to hopefully improve the tumor take in nude mice, which is low for these cancers as compared with colon carcinomas and melanomas (2).

Recent studies of different human tumors have shown that the site of implantation can affect whether distant metastases are formed (9–14), although it has yet to be determined how the tissue environment affects the tumor cell phenotype. For the MDA-MB-435 breast carcinoma, the m.f.p. supported local growth, invasion, and metastases, while a lower incidence of metastasis was seen from s.c. tumors. In contrast, MDA-MB-231 tumors also grew in the m.f.p. but produced fewer macroscopic metastases, as compared with MDA-MB-435 tumors. Thus, whereas the m.f.p. can promote the growth of breast carcinomas, it does not necessarily induce expression of the metastatic phenotype (or tumorigenicity) in all tumors.

Metastases were found in the lungs and lymph nodes of most
mice with MDA-MB-435 m.f.p. tumors, and the numbers of lung metastases were related to the tumor size. However, i.v. injection of a bolus of MDA-MB-435 cells produced few lung colonies. Possible explanations for this are that dissemination via the lymphatics is the principal route for MDA-MB-435 cells or that the formation of lung metastases required millions of cells to be shed from the m.f.p. tumors, over a number of weeks. A previous study using mouse mammary tumors provides similar examples of tumors that were metastatic in the autochthonous host (i.e., growing in the mammary tissue) but that had poor lung-colonizing potential following single i.v. injections of 10^6 cells into syngeneic mice (27).

Selection of tumor cell populations with increased metastatic properties can be achieved by isolating the metastases and establishing new variants of a tumor (11, 28, 29). In this study the lung metastasis-derived cell lines showed a modest increase in numbers of lung metastases formed, compared with the original MDA-MB-435 cell line, which was highly metastatic from the m.f.p.. None of the cell lines derived from metastases from m.f.p. tumors showed enhanced lung colonization potential after i.v. injection. Only cell lines from lung colonies, resulting from i.v. injection of single cells or from tumor cells grown on Cytodex beads, showed enhanced lung-colonizing properties (Table 3). We can conclude from this that the tumor cell properties selected for by the process of metastasis from m.f.p. tumors may not be exactly the same as those required for successful formation of experimental lung metastases by the human breast carcinoma cells (28).

Lymph nodes and lungs are the most common sites for metastasis of human tumors in nude mice. Finding brain metastases in some mice with MDA-MB-435 tumors adds to the value of this cell line for a model of metastatic breast carcinoma. Occasional brain metastases were large enough to be grossly visible, and the 435-BR1 cell line was established from one of these. On rejection into the m.f.p. of mice, this cell line produced few lung metastases, compared with the original or lung metastasis-selected cell lines. Current studies are comparing the 435-Lung and 435-BR1 cell lines to investigate the cellular basis of the apparent organ preference of metastasis, in terms of differential adhesion to extracellular matrices or capillary endothelial cells (30, 31) and responses to organ-derived growth factors (30, 32, 33).

Metastatic breast carcinoma is a major cause of death in North America and Western Europe, yet to date few model systems are available for experimental studies of this important disease. In this initial report, we describe the growth of human breast carcinoma cells in the m.f.p. of nude mice and the dissemination of metastases to several different organs. This model system could be an important element in further studies on the cellular and molecular basis of the metastatic phenotype of human breast carcinoma.

ACKNOWLEDGMENTS

We thank Patherine Greenwood for preparing the manuscript.

REFERENCES


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