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Metastatic Potential of Hyperplastic Alveolar Nodule Derived Mouse Mammary Tumor Cells Following Intravenous Inoculation*

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Abstract—*A comparison was made of the* in vivo *growth potential of tumor cells derived from mouse mammary adenocarcinomas arising in premalignant outgrowth lines D1 and D2. Twenty-one tumors were dissociated and cultured as cell monolayers* in vitro. *Three to four* days *later* 50,000–100,000 *cells were inoculated intravenously into syngeneic female BALB/c mice. Primary cultures of tumor cells which arose in D2 HAN outgrowths varied considerably in their ability to form tumor nodules in the lungs of syngeneic mice* 4 weeks *post-inoculation. An increase in either the number of cells inoculated or in the length of* in vivo *incubation influenced the extent of tumor growth observed in the lungs. Primary cultures of tumor cells which arose in D1 HAN outgrowths inoculated at the same cell number rarely formed tumor nodules in the lungs* 4 weeks *post-injection. If, however, twice as many cells were introduced, a small number of large nodules were observed within* 4 weeks. *An epithelial cell line (WAZ-2T) isolated from D1 tumor tissue produced small lung nodules by* 4 weeks, *but larger more numerous nodules arose when* in vivo *incubation was extended an additional* 3–6 weeks. *This study indicates that primary D2 tumor cells have a variable but higher degree of metastatic potential than primary D1 tumor cells following intravenous inoculation. Tumors which develop in the D2 and D1 premalignant lines may be useful for studies of chemotherapy and cell latency of tumor metastases and the identification of the specific cell subpopulations involved in these events.*

INTRODUCTION

A CLEAR understanding of both the host and tumor characteristics involved in tumor metastasis is of vital importance in determining new and successful means for treatment of human cancer. Toward this end an increasing number of investigators have studied metastasis using animal model systems [1–3]. The study by Tarin and Price [3] utilized spontaneous mammary tumor virus (MTV) induced tumors and showed that these tumors vary in metastatic potential once they are introduced into the vascular system. The usefulness of mouse mammary tumors for studies of metastasis has also recently been demonstrated by Shewell and Thompson [4] in therapy-related experiments using hyperbaric oxygen.

In the present study we have investigated the use of non-MTV induced mammary tumors as a source of malignant epithelial cells for studies of hematological metastasis. We believe that the lack of virus production by these cells provides a cell system more closely approximating human cancer. We have used primary cultures of mammary cells to increase cell viability, to eliminate interfering host cells which are released with the initial tumor suspensions and to minimize the effect of tumor necrosis on growth potential. The tumors we used arose in two transplantable lines of hyperplastic alveolar nodules (HAN) developed in BALB/c mice by Medina[5]. One of these lines, D2, has a high spontaneous rate of malignant transformation while the other line, D1, transforms at a much lower rate [5]. We were particularly interested in whether a difference in metastatic potential existed between D1 and D2 cells after both HAN tissues had trans-

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formed to malignancy. We have accumulated data on the extent of organ colonization by individual tumors derived from D1 and D2 tissue and discuss the relevance of this system to human disease.

MATERIALS AND METHODS

Tumors and cell cultures

Spontaneous mammary adenocarcinomas which arose in D1 and D2 transplants [5] were used for all experiments. Tumors were removed and dissociated using collagenase type I (Sigma Chemical, St. Louis, MO) as previously described [6]. The dissociated cells were plated at 10^5 cells/cm^2 into 75 cm^2 culture flasks (Falcon Plastic, Oxnard, CA) in the presence of Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (Flow Laboratories, Rockville, MD), 10 μg/ml insulin (Sigma Chemical), 100 μg/ml streptomycin (Sigma Chemical) and 50 μg/ml penicillin (Calbiochem.-Behring Corporation, La Jolla, CA). We also used the WAZ-2T cell line, originally isolated from a tumor which arose in a D1 outgrowth [7].

Tumor cell inoculations

Cultures nearing confluence were treated with STV (saline containing 0.05% trypsin and 0.025% Versene) to produce cell suspensions consisting of primarily single cells. The cells were washed and resuspended in serum-free DME or balanced salts solution. From 50,000 to 100,000 cells were injected into the lateral tail vein of female, syngeneic BALB/c mice. Cell viability in the cell suspension after the last inoculation was completed was always between 70 and 90% (trypan blue exclusion). Four to eight weeks following inoculation the mice were sacrificed and the internal organs examined macroscopically. Tumor nodules were found only in the lungs. All lungs (with and without apparent modules) were fixed in Bouin's fixative overnight, then washed with 70% ethanol and tumor nodules counted using a dissecting microscope (20×). Lung nodules of the grade I classification (see below) were often verified as tumor tissue by histological analysis.

Histology

Fixed specimens of the lungs and original tumors were embedded in paraffin and sectioned by standard techniques. Sections were stained with hematoxylin–eosin.

Scoring of tumor nodules

We have utilized a scoring system which is a modification of the grading scale used by Tarin and Price (Table 1) [3].

RESULTS

Primary tumor cells derived from D2 HAN outgrowths

Seven tumors which spontaneously arose in transplanted D2 outgrowths were dissociated, cultured and inoculated as described in the Materials and Methods. The cultured tumors exhibited an epithelial morphology and possessed growth characteristics typical of mammary tumor cells in monolayer culture [8].

All mice injected intravenously with cells from this initial group of D2 tumors were sacrificed after 4 weeks. Tumor nodules were apparent only in the lungs of these animals (macroscopic examination). One of the 7 tumors studied D2T3 produced a significant number of tumor nodules in the lungs, but the remaining 6 tumors were capable of only limited growth in the lungs within this time period (Table 2). Using the grading system outlined in the Materials and Methods, only the D2T3 cells could be scored as having a high metastatic potential.

To determine whether the time of examination influenced the scoring of metastatic

Table 1

LOW	Grade 0 = no tumor nodules present. Grade I = less than 10 nodules and nodular diameter < 1 mm.
MEDIUM Metastatic potential	Grade II = less than 5 nodules with some nodular diameters > 1 mm. Grade III = greater than 10 nodules but most of small diameter (< 1 mm).
HIGH	Grade IV = greater than 20 nodules of varying sizes but with greater than half larger than 1 mm. Nodules distinct. Grade V = many nodules (> 30) and a mixture of tumor sizes with a majority larger than 1 mm. Nodules intermingling. Grade VI = massive tumor growth, little identifiable lung tissue remains.

Table 2. Lung nodules from tumor cells which developed in D2 HAN outgrowths

Tumor	Grade of lungs*	Metastatic potential
D2T1	0, 0, 0, I, I, I	Low
D2T2	0, I, I, I, I, III, III	Low/Medium
D2T3	III, IV, IV, IV, IV, IV, IV	High
D2T4	I, I, I, I, III	Low
D2T5	0, 0, 0, I, I	Low
D2T6	0, 0, 0, 0, 0, 0	Low
D2T7	0, 0, 0, 0, I	Low

*50,000 cells inoculated per mouse and mice were examined after 4 weeks. Each numeral represents the lungs of one mouse.

potential, 4 additional D2 tumors were injected using 50,000 cells per mouse and examined at 4 and 8 weeks. Two of the tumors studied in this group (D2T10 and D2T13) were initially scored as having a low metastatic potential (4 weeks), however by 8 weeks both had progressed to grades III through VI with several early (pre-examination) deaths (Table 3). The other 2 tumors of this group (D2T11 and D2T12) were non-metastatic or weakly metastatic even after 8 weeks incubation *in vivo*.

An increase in the number of cells per mouse also had an effect on the scoring of metastatic potential. Two tumors (D2T8 and D2T9) inoculated at 100,000 cells per mouse produced extensive tumor growth after 4 weeks (Table 3). The extent of tumor growth found in these mice within 4 weeks (grades IV, V and VI) was only rarely observed in mice inoculated with 50,000 cells (see Tables 2 and 3).

The histology of the primary tumors which arose in D2 HAN tissue was consistent with mammary adenocarcinoma types A and B; as described by Dunn [9], the distinction between these 2 types is often arbitrary. Regular and irregular acinar structures were present in these tumors, as well as numerous mitotic figures (Fig. 1). The tumor cells were very basophilic, with large nuclei containing multiple nucleoli. Encapsulation of the tumors was poorly developed. All lung nodules resulting from D2 tumor cells were readily recognizable as mammary adenocarcinomas and were histologically identical to the primary tumors (Fig. 2).

Primary tumor cells derived from D1 outgrowths and studies with the WAZ-2T cell line

Eight D1 tumors were cultured *in vitro*. The first 3 tumors studied were injected using 50,000 cells per mouse and all 3 failed to produce lung nodules within a 4 week incubation period (Table 4). A second group of 5 D1 tumors were studied and injected at 50,000 and/or 100,000 cells per mouse. The data from these incubations are also presented in Table 3 (D1T4–D1T8). Using 50,000 cells and 4 weeks incubation only 1 tumor nodule was produced (grade I). Two other mice inoculated with 50,000 cells died within 8 weeks though the exact cause of death was not determined. After 8 weeks, 1 additional mouse injected with 50,000 cells was found to have grade II lung nodules.

As was found with the D2 tumor cells, an increase in the number of D1 tumor cells inoculated resulted in an increase in the number of mice with lung nodules. Unlike the nodules produced by D2 tumor cells, however, the D1 nodules were few in number but usually greater than 1 mm in diameter. The grade of tumor produced was thus much lower with D1 tumor cells, and a longer incubation period *in vivo* had less effect on the grade of tumor produced than with some D2 tumor cells. Nonetheless, 4 out of 5 D1 tumors inoculated at 100,000 cells did form grade I or II nodules in the lungs.

Histological examination of D1 tumor nodules revealed a tissue pattern identical to primary D1 tumors (Fig. 3). These type B mammary adenocarcinomas were quite different from D2 tumors, in that they were

Table 3. Lung nodules from tumors arising in D2 tissue using an increased number of cells or longer incubation period

Tumor	No. cells inoculated	Grade of lungs per mouse inoculated Examined at: 4 weeks	8 weeks	Metastatic potential Examined at: 4 weeks	8 weeks
D2T8	100,000	V, V, V, VI, VI	—	High	—
D2T9	100,000	IV, IV, IV, V	—	High	—
D2T10	50,000	0, 0	III, IV, V and 2 deaths	Low	High
D2T11	50,000	0,0	0, 0, 0, 0, 0	Low	Low
D2T12	50,000	0, I	0, 0, 0, I, I, I	Low	Low
D2T13	50,000	0, III	IV, V, VI, VI	Low/Medium	High

Table 4. Lung nodules from tumor cells which developed in D1 HAN outgrowths

Tumor	No. cells inoculated	Grade of lungs per mouse inoculated Examined at: 4 weeks	8 weeks	Metastatic potential Examined at: 4 weeks	8 weeks
D1T1	50,000	0, 0, 0, 0, 0	—	Low	—
D1T2	50,000	0, 0, 0, 0, 0, 0	—	Low	—
D1T3	50,000	0, 0, 0, 0, 0	—	Low	—
D1T4	50,000	0, I	0, 2 deaths	Low	Medium
	100,000	II, II, II	0, 1 death	Medium	Medium
D1T5	50,000	0, 0	0, II, 1 death	Low	Medium
	100,000	0, II	0, II, II	Low/Medium	Medium
D1T6	100,000	0, 0, 0, 0, I	—	Low	—
D1T7	100,000	0, 0, II	0, II, II	Low	Medium
D1T8	100,000	0, 0, 0	0, 0, 0	Low	Low

made up primarily of epithelial "cords" with few or no acini present. The connective tissue around each epithelial cord of cells was well developed and the tumor cells were quite basophilic.

The WAZ-2T cell line, which was originally isolated from a primary D1 tumor, produced lung nodules within 4 weeks of inoculation (Table 5). All nodules formed by WAZ-2T cells were adenocarcinomas of type B, similar to the primary tumors originating in D1 tissue. Nodule-containing lungs of WAZ-2T inoculated mice were scored as grade I in classification and all nodules were less than 1 mm in diameter. Studies which we have recently published elsewhere [7] indicate that longer incubation *in vivo* results in much larger and more numerous WAZ-2T tumor nodules (grades V and VI).

Table 5. Lung nodules produced by WAZ-2T cells

Experiment	Grade of lungs*	Metastatic potential
1	0, I, I, I, I, I	Low
2	0, 0, 0, 0, I, I	Low
3	0, 0, I, I	Low

*All mice were inoculated with 50,000 cells and examined after 4 weeks.

DISCUSSION

The mouse mammary adenocarcinoma has been extensively studied and a great deal has been learned about the biological [10] and immunological [11] features of these tumors. While mammary tumor metastasis is seldom involved in the mortality of these mice spontaneous metastases do occur [12]. We believe that primary mammary tumors provide a useful source of malignant epithelial cells, in contrast to the more commonly studied mesenchymal cell systems, for studies of tumor metastasis.

We have shown that spontaneous tumors derived from hyperplastic alveolar nodules will form tumors in the lungs of syngeneic mice. In general our results support those of Tarin and Price [3] in that we also found a substantial diversity in metastatic potential amongst mouse mammary adenocarcinomas, even when derived from a common source of premalignant tissue. We believe, however, that our techniques more closely reflect natural events since Tarin and Price [3] included newborn calf serum in the cell inoculum and used a very large (10^6) cell dose for the intravenous inoculations in their experiments. In our study we injected a much lower tumor cell dose of 50,000–100,000 cells per mouse in medium free of all calf serum antigens. Using these techniques we observed substantial tumor growth in the lungs in less time than did Tarin and Price, with little variance between mice inoculated with a single tumor suspension.

Within the group of tumors resulting from D2 tissue, a range of metastatic potential existed when lungs were examined after 4 weeks. For example, D2T3 cells were moderately aggressive (grade IV), while D2T6 cells were entirely non-tumorigenic within the same time period. Further studies using a higher cell inoculum or longer incubation period *in vivo* showed that at least some of the diversity in metastatic potential of D2 tumors was dependent upon these 2 variables. The appearance of early tumor nodules, however, did not necessarily mean that longer incubation *in vivo* would result in larger or higher grade tumors (e.g. D2T12).

The diversity of cell subpopulations in HAN tissue [13, 14] and in various non-mammary tumors [15, 16] led us to compare the growth

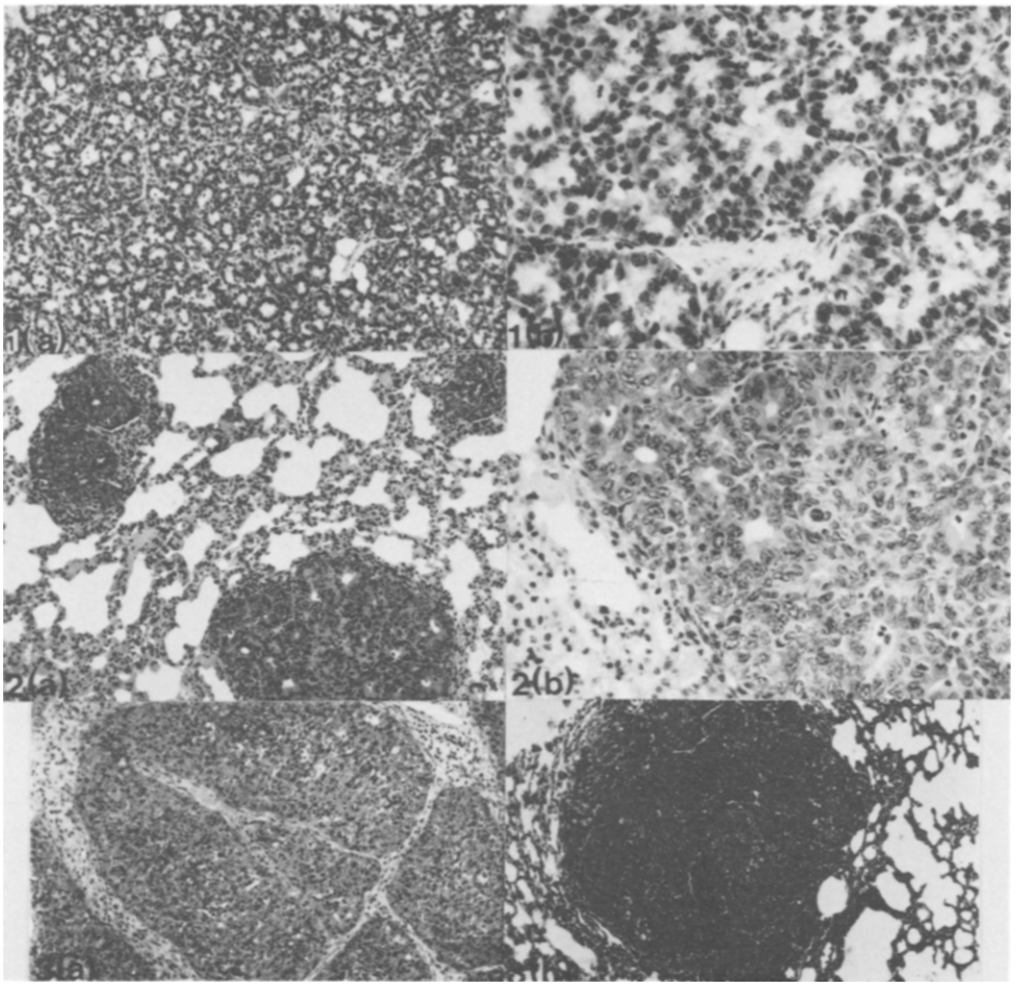

Fig. 1. (*a*) *Mammary adenocarcinoma from D2 HAN tissue. Numerous acinar structures are present with very little connective tissue.* (*b*) *Higher magnification shows the numerous mitotic cells observed in these tumors.* 128×; 320×.

Fig. 2. (*a*) *Lung nodules formed by mammary tumor cells derived from D2 tissue following intravenous inoculation.* (*b*) *Glandular elements of lung nodules are readily apparent as well as many mitotic cells and distinct lack of well developed encapsulation.* 128×; 320×.

Fig. 3. (*a*) *Mammary adenocarcinoma from D1 HAN tissue with cords of epithelium surrounded by well developed connective tissue.* (*b*) *Lung nodule formed by D1 tumor cells.* 128×; 128×.

potential of D1 and D2 tumor cells. We found that most D2 tumors injected at 50,000 cells/mouse produced lung nodules within 4 weeks, but under similar conditions nodules were much less frequently formed by cells from primary D1 tumors. An equally significant difference in metastatic potential was also observed when 100,000 cells were inoculated. This is an interesting difference between tumors of similar preneoplastic origin which may be related to variations in the subpopulations present in the original HAN tissues [14]. While D1 tumor cells did produce tumor nodules at the higher cell inoculum, these were usually limited to 1–3 large nodules per mouse which may have resulted from cell clumps formed at this higher cell density. These large nodules were occasionally lethal within 8 weeks. In contrast, D2 tumor nodules were of many different sizes at 4 weeks (<1 mm) and greater in number. The number of nodules formed by some D2 tumors also increased substantially between 4 and 8 weeks; a finding not observed with D1 tumor cells. These facts lead us to believe that D2 tumors contain many more cells capable of metastatic growth than do D1 tumors.

If our assumptions about D1 tumor nodules are true, they are in agreement with an earlier study[17] using C3H mammary tumor cells in which single cells were found to be non-metastic. Thompson[17] was further able to show that aggregated C3H tumor cells would form lung nodules. We have evidence from ^{125}IUDR labeling studies that the absence of growth by low doses of D1 tumor cells is not due to a lack of initial cell arrest in the lungs (data not shown).

An additional question that we wished to address in this study was whether a period of growth quiescence occurs in the lungs of mice inoculated with mammary tumor cells. We have found that a period of either very slow growth or quiescence does occur, as shown by D2 tumors 10 and 13 and by the D1 tumor cells of the WAZ-2T cell line [7]. This finding indicates that the mouse mammary carcinoma may be useful for studies of treatment modalities for latent tumor cells which very likely are responsible for many of the relapses observed in human cancers [1]. In this regard several of the currently popular tumor systems may be less useful due to the extreme and rapid tumorigenicity of the cells involved.

We believe that the numerous mammary tumor cell lines available [7, 18, 19], in conjunction with the multiple sources of primary mammary tumors, make the mouse mammary system attractive for further studies of tumor metastasis. Indeed, the study of slow growing, latent tumor cells, such as found in some D2 tumors and the WAZ-2T cell line, may be more relevant to human disease than currently studied model systems.

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