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In Vitro Characteristics of Metastatic Variant Subclones of Restricted Genetic Origin

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We have studied several metastatic variant cell lines derived from a common clonal origin and their transformed and untransformed parental cell lines. A number of in vitro characteristics were examined for each tumor line and these properties were correlated with the ability of the tumor cells to form pulmonary nodules in an experimental metastasis assay.

Direct correlations with metastatic behavior in the lung colony assay were found to exist with the amount of cell-bound Concanavalin A and the procoagulant activities of cell lysates. In vitro parameters that did not correlate with the metastatic phenotype were: population doubling times in culture, saturation density achieved in culture, the number of colony-forming cells shed from confluent cultures, rates of cellular attachment to homotypic or heterotypic cell monolayers, plasminogen-activator production and procoagulant activity produced in serum-free conditioned medium.

Key words: metastatic variants, in vitro correlates, rat

Metastasis is a clinically important event in tumor pathogenesis. It is a complex phenomenon involving numerous steps: release of tumor cells from the primary tumor, invasion and circulation through the vasculature, trapping and arrest, extravasation, and survival and proliferation of the micrometastases in the secondary sites. The population of cells comprising a primary tumor is often heterogeneous with respect to metastatic potential [1]. The process of metastasis requires cells that can disseminate, survive, and proliferate in a new tissue environment.

We have developed a series of metastatic variants from a clone cell line that was itself derived from an epithelioid cell line of hepatic origin. This original cell line was initiated from a BD-IV inbred rat and chemically transformed in vitro [2, 3]. The cloned parental cell line metastasizes to the lungs and exhibits a stable homogeneous metastatic phenotype that is unaffected by in vitro passage for nine months or by in vitro subcloning procedures [4]. Partial ascites adaptation of the parent line introduced metastatic heterogeneity into the cell population allowing for subsequent isolation of the metastatic variants. Scanning electron microscopy of the variants grown in tissue culture reveals a varying of surface morphologic characteristics that correlates with their metastatic ability [4].

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In this paper, we report the results of studies on several in vitro characteristics of the metastatic variants and correlate these with the in vivo metastatic phenotype. Of the characteristics examined, few correlated well with the metastatic capabilities observed in vivo, although several were associated with the malignant cellular phenotype.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The metastatic variant cell lines examined in this work were derived from a clonal isolate, RT7-4b. This clonal cell line was initiated from a primary cell culture of metastatic lung nodules that developed following intravenous injection of the dimethylnitrosamine transformed cell line, IAR6-1-RT7. The hepatic cell line IAR6-1-RT7 and its untransformed parental cell line IAR6-7 [2, 3] were donated by Dr. R. Montesano (International Agency for Research of Cancer, Lyon). Introduction of metastatic heterogeneity into the RT7-4b clone by in vivo ascites passage and the subsequent isolation of metastatic variants are described in a previous paper [4]. The metastatic variant subclones studied in this work were chosen to represent the full range of metastatic behavior observed, and are, in order of increasing metastatic ability in the lung colony assay: RT7-4bA, RT7-4bM, RT7-4bL, and RT7-4bE (Table I). Dermal fibroblast cell cultures were used at the third passage following initiation of primary cultures from a day-old BD-IX rat [5].

Cell cultures (initially seeded at 5×10^3 cells/sq cm) were subcultured weekly at a 1:4 split ratio and maintained at 37°C in a humidified atmosphere of 7% CO_2 in air. The cell lines were grown in Ham's nutrient mixture F-12 (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 units/ml), and streptomycin (100 μg/ml). This was designated complete medium. Maintenance and subculture procedures were as previously described [4]. Other media and buffers used were: Eagle's minimum essential medium (MEME; Gibco) supplemented with 5% FBS; Tyrode's balanced salt solution (TBSS); Ca^{++}, Mg^{++}-free Tyrode's balanced salt solution (CMF); Ca^{++}-, and Mg^{++}-free Puck's saline G; and bivalent cation solution (BCS) (PBS containing 1.5×10^{-3} M $MgSO_4 \cdot 7\ H_2O$, 1.4×10^{-4} M $CaCl_2$, 1.0×10^{-4} M $MnCl_2 \cdot 6\ H_2O$).

Population Doubling Times

Monodispersed cells were seeded at 10^5 cells per tissue culture dish (21 sq cm growth area) in complete medium. The cultures were fed every other day by replacing the medium with fresh complete medium. The numbers of cells present per tissue culture dish were enumerated daily. Cells were harvested by trypsinization and counted using a hemocytometer. The means of triplicate cultures were plotted and the population doubling time was calculated [6].

Spontaneously Detached Cells From Monolayer Cultures

The number of colony-forming cells shed from confluent monolayer cultures (21 sq cm growth area) into the overlying medium during a 48 h incubation period was measured. The cultures were fed, and 48 h later, the overlying 5 ml of medium was gently removed and placed in a disposable plastic test tube. Aliquots (1 ml) were removed from the tubes after agitation to disrupt cell clumps, diluted in 10 ml of complete medium, and then transferred to 100 × 20-mm tissue culture dishes. Medium containing spontaneously detached cells was harvested from triplicate cultures, and duplicate dilutions were plated for each sample. The cultures initiated from these cells were incubated undisturbed at 37°C for

TABLE I. Pulmonary Tumor Colonies Obtained Following Intravenous Injection of Variants Isolated From the Partially Ascites-Adapted Cell Population 3XAA-RT7-4b

Cell line	Number of rats injected	Median no. of pulmonary tumor colonies	Mean no. of pulmonary tumor colonies ± SD[a]	Range	Significance[b]
Parent clone RT7-4b	24	98	110 ± 58	46–227	
3XAA-RT7-4b– A	10	8	12 ± 11	0–29	0.0001
E	9	406	396 ± 176	195–694	0.0001
L	10	204	205 ± 60	115–286	0.0002
M	9	61	89 ± 62	23–203	NS[c]

[a]Mean number of pulmonary tumor colonies per rat ± standard deviation (left pulmonary lobe only). Obtained by averaging the results from a minimum of two different sets of five animals.
[b]Probability of difference from pooled data for parent clone RT7-4b (Mann Whitney U-test).
[c]NS: No significant difference between parent clone RT7-4b and the variant ($P = 0.05$).

6 days. The medium was then removed from the cultures, and these were fixed, stained, and the number of colonies present were enumerated.

Iodination

Fibrinogen (Sigma) and Concanavalin A were radiolabeled with ^{125}I (New England Nuclear) using the chloramine-T technique [7]. The reaction products were removed using a 16% sucrose solution wash. These were layered on disposable Sephadex G-25 columns and eluted with 0.05 M phosphate buffered saline, pH 7.0. The iodinated protein eluted as the first peak of radioactivity. Specific activities of the ^{125}I Con A were as follows: experiment 1, 4×10^5 cpm per μg; experiment 2, 1×10^5 cpm per μg; experiment 3, 1.5×10^4 cpm per μg.

Plasminogen Activator Production

Fibrinogen type V from canine blood was purchased from Sigma and subsequently purified to fibrinogen II [8]. Following iodination, 0.1 ml of ^{125}I-fibrinogen was spread over the base of each well of a Falcon 3008 plate. The plates were dried at 45°C for 5 days, and then sterilized by UV irradiation. The fibrinogen was converted to fibrin by incubation with MEME containing 5% thrombin-enriched FBS at 37°C for 3 h. The wells were then washed 3 times with serum-free MEME and stored overnight at 4°C.

Cell cultures were trypsinized, the trypsin neutralized by the addition of complete medium, the cells pelleted, and washed with serum-free Ham's F-12 medium. The cells (10^5) were added to each well in serum-free medium and incubated at 37°C for 2 h. The medium was then adjusted to 5% FBS and incubation was continued for a further 6 h. At the end of this incubation period, the medium was removed and the wells washed three times with serum-free medium. Test medium consisting of 1 ml serum-free Ham's F-12 with 3 μg/ml plasminogen (Sigma) was added, and the cultures were incubated for 24 h at 37°C. To evaluate plasminogen-independent release, replicate cultures were incubated in plasminogen-free medium. A trypsin digest was used to obtain the total number of protease-releasable ^{125}I counts per minute per well. Quadruplicate wells were examined for each test point.

Rate of Cell Adherence to Monolayers of Homotypic and Heterotypic Cells

Cultures of IAR6-7, RT7-4b, and rat fibroblasts were trypsinized, and equal aliquots were added to the wells of Falcon 3008 multiwell dishes in complete medium. The plates

were incubated for 3 days until confluent monolayers had formed. Cell lines to be tested for their attachment rates to the monolayers were seeded in T-75 flasks at 3×10^6 cells per flask. After an overnight incubation, the medium was replaced with 10 ml of complete medium containing 1 μCi per ml ^{125}IUDR (Amersham). Following incubation at 37°C for 24 h, the medium was aspirated and the monolayers were washed twice with CMF. The cells were removed by incubation in a solution of 0.1 mM EGTA in Ca^{++}- and Mg^{++}-free Puck's saline G. The cells were pelleted, washed with CMF, and counted. One hundred μl aliquots of 10^5-labeled cells (3–5 cells per cpm) were then added to each target monolayer-containing well. Prior to the addition of labeled cells, the medium in the wells was replaced with 0.5 ml of fresh complete medium. The cultures were incubated at 37°C for 30, 60, or 120 min. The medium was then aspirated, and target monolayers were washed twice with MEME-containing 2% FBS. The monolayers were lysed using a 2-h incubation in 1 ml of 2% Nonidet P-40 at 37°C. The amount of cell-bound ^{125}IUDR present per monolayer at each time point and the cell-associated ^{125}I counts added per well in the original cell suspensions were determined. The percentage of originally added cells adhering to the monolayers was expressed as a percentage of total cell-associated counts added to the wells. Quadruplicate samples were examined for each point.

Cellular Binding of Con A

Cells to be studied were grown to confluency in Falcon 3008 multiwell plates. In addition to the four metastatic variant cell lines, the parent clone, RT7-4b, the transformed cell line from which RT7-4b is derived, IAR6-1-RT7, and the "normal" hepatocyte cell line from which IAR6-1-RT7 is derived, IAR6-7, were studied. After the cells were grown to confluency, the medium was removed, the monolayers washed twice with BCS and they were allowed to equilibrate to temperature for 10 min. The last BCS wash was replaced with 1 ml BCS containing ^{125}I-Con A or ^{125}I-Con A with α-methyl-D-mannopyranoside. The amount of Con A added per well was as follows: experiment 1, 25 μg/well; experiment 2, 27 μg/well; and experiment 3, 12 μg/well. α-Methyl-D-mannopyranoside concentrations of 0.7 mM, 12.5 mM, and 125.0 mM were used. The plates were then incubated at the temperatures indicated for 120 min (experiments 1 and 2) or 180 min (experiment 3). At set times during the incubation with Con A, sample wells were harvested. The incubation mixtures were removed, the monolayers washed four times with BCS and lysed using a 2-h incubation in 1 ml 2% SDS at 37°C. Con A bound per well was calculated from the ^{125}I counts present in the lysate. Cell numbers were obtained from trypsin-harvested replicate wells. Each data point represents the average of four replicate wells.

Cell-Associated Procoagulant Activities

Procoagulant activities were assayed in canine platelet-poor plasma (PPP) using modifications of the single-stage prothrombin clotting time assay. Canine platelet-poor plasma congenitally deficient in factor VII was donated by Dr. W. Jean Dodds of the Division of Laboratories and Research, New York State Department of Health, Albany, New York.

Standard dilution curves of rabbit brain thromboplastin clotting times in these plasmas yielded straight-line plots on log-log coordinates. Standard thromboplastin procoagulant curves were prepared each time unknown procoagulant activities were assayed and were used to determine activities in terms of thromboplastin equivalence units. Conditioned serum-free medium and cell lysates were examined for procoagulant activity. Conditioned medium was obtained from 6-h incubations with cells in late log phase of growth. Cell lysates were prepared by four freeze/thaw cycles. A macroprothrombin clotting time assay

TABLE II. In Vitro Growth Characteristics of the Parent Cell Line and the Metastatic Variants

Growth characteristic	RT7-4b	RT7-4bA	RT7-4bE	RT7-4bL	RT7-4bM
Population doubling time (hours)	12.9	11.1	12.8	13.6	10.2
Saturation density[a] ($\times 10^5$)	29.7 ± 6.3	29.1 ± 6.6	32 ± 2.5	28 ± 2.5	29.3 ± 1.2
Number of colony-forming cells shed into the medium in 48 h[b]	25 ± 3	98 ± 8	50 ± 23	232 ± 91	20 ± 12

[a]Number of cells in 21 sq cm of growth area at saturation density. Mean ± standard deviation of triplicate cultures.
[b]Mean number of colony-forming cells present per ml of overlying medium.

was used in which 1 ml of test sample was added to 1 ml of citrated PPP. One milliliter of 0.025 M $CaCl_2$ was then added, and timing started immediately. Timing was stopped when a visible fibrin clot appeared. The clotting times were assayed in triplicate and were performed in Falcon 2001 tubes. All reagents, tubes and pipets were prewarmed to 37°C. All dilutions were made in imidazole-buffered saline, pH 7.4 (Sigma). The results were expressed in thromboplastin equivalence units of activity released by 10^6 cells into serum-free medium or from 10^5 lysed cells.

RESULTS

Behavior of the Metastatic Variants in the Lung Colony Assay

The metastatic phenotypes of the parent clone and the metastatic variants are presented in Table I. The metastatic abilities of the variant subclones in the lung colony assay are as follows: RT7-4bE > RT7-4bL > parent clone RT7-4b = RT7-4bM > RT7-4bA. The metastatic variants RT7-4bE, RT7-4bA, and RT7-4bL are significantly different from the parent clone RT7-4b (Table I). The metastatic capabilities of the variants are also significantly different from one another ($P < 0.001$, Mann Whitney U test, data not shown).

In Vitro Growth Characteristics

In vitro growth characteristics of the parent line and the variant subclones are presented in Table II. The metastatic variants and the parent line RT7-4b exhibited similar population doubling times. The saturation densities of these cell lines were also similar, ranging from 28×10^5 cells per 21 sq cm of growth area for RT7-4bL to 32×10^5 cells per 21 sq cm of growth area for RT7-4bE.

The number of colony-forming cells shed into the overlying medium from completely confluent cultures during a 48-h incubation period was studied. A large variation was found in the number of colony-forming cells shed between the individual variant cell lines. In all cases, relatively few cells were shed by these cultures in comparison with data reported for other tumor cell lines [12].

Plasminogen Activator Production

The production of plasminogen activator by the parent clonal cell line and the metastatic variants was studied using cellular digestion of an ^{125}I-fibrin substrate (Table III). Plasminogen-dependent digestion was induced by cell lines IAR6-7, IAR6-1-RT7, RT7-4b and RT7-4bA. The other metastatic variants at the same cell density failed to produce sig-

nificant levels of plasminogen activator. The cell line IAR6-1-RT7 exhibited an additional plasminogen-independent protease activity.

Cellular Attachment Rates to Monolayers of Homotypic and Heterotypic Cells

The rate of attachment of the parent clonal cell line and the metastatic variants to monolayers of cells was examined in vitro. The target cell monolayers utilized were: the syngeneic untransformed hepatic cell line IAR6-7, the homotypic parent cell line RT7-4b, and early passage BD-IX rat fibroblasts (Table IV).

All of the cell lines adhered faster to the fibroblast monolayers than to the hepatocyte monolayers. Adherence occurred least rapidly to IAR6-7, more rapidly to RT7-4b, and most rapidly to the fibroblast cell line. At the first time point observed, 30 min, the attachment of the variants to the homotypic parental cell line RT7-4b was as follows: RT7-4bL = RT7-4b > RT7-4bM ≫ RT7-4bE = RT7-4bA, while attachment to the fibroblast was as follows: RT7-4bM = RT7-4b = RT7-4bL > RT7-4bE > RT7-4bA. By 60 min, differences between the subclones in the percentage of cells adhering were less pronounced.

Procoagulant Activity of the Metastatic Variants

Procoagulant activity of the metastatic variants was determined in conditioned serum-free medium and for cell lysates. Normal canine and factor VII-deficient canine PPP were used as substrates (Table V).

Little procoagulant activity was released into conditioned serum-free medium compared with that present in the cell lysates. However, RT7-4bA released higher levels of procoagulant activity into conditioned medium than did the other variants. Very little factor VII-independent procoagulant activity was found in the serum-free media conditioned by the metastatic variants.

The relative procoagulant activities of the cell lysates towards normal PPP were as follows: RT7-4bL > RT7-4bE > RT7-4bA > RT7-4bM. The variant, RT7-4bM, exhibited a higher level of factor VII-independent than factor VII-dependent procoagulant activity,

TABLE III. Plasminogen Activator Production by the Parent Cell Lines and the Metastatic Variants

Cell line		Percentage of nonspecific ^{125}I-fibrin release (CPM ± SD)[a]	Percentage of specific ^{125}I-fibrin release (CPM ± SD)[b]
IAR6-7		0.0 (1,496 ± 53)	48 (33,897 ± 6,174)
IAR6-1-RT7		18.0 (12,316 ± 872)	21 (26,319 ± 1,764)
RT7-4b		0.0 (2,008 ± 421)	18 (13,907 ± 1,073)
RT7-4bA		2.5 (3,527 ± 258)	17 (14,799 ± 2,477)
RT7-4bE		0.0 (1,850 ± 85)	1.5 (2,878 ± 106)
RT7-4bL		0.0 (2,435 ± 157)	3.0 (4,516 ± 340)
RT7-4bM		0.0 (1,296 ± 73)	0.0 (1,327 ± 135)
Total release[c] CPM ± SD	67,766 ± 11,187		
Bkg release[d] CPM ± SD	1,817 ± 217		

[a]Percentage of cell-induced plasminogen-independent release.
[b]Percentage of cell-induced plasminogen-dependent release (plasminogen activator production).
[c]Total release of ^{125}I-fibrin substrate by trypsin.
[d]Background release from ^{125}I-fibrin substrate in the presence of serum-free medium and plasminogen only.

an activity similar to that reported for cancer procoagulant A. The other subclones all exhibited factor VII-dependent procoagulant activity consistent with normal tissue thromboplastin activity.

Cellular Binding of Con A

The amount of Con A binding to the various cell lines is presented in Table VI (experiment 1). The total amount of Con A bound in 2 h at 37°C is presented and was greatest for RT7-4bE > RT7-4bL ⩾ RT7-4bM = RT7-4b > IAR6-1-RT7 > RT7-4bA > IAR6-7. Similar binding kinetics were found for Con A binding at 4°C (experiment 2) and at 30°C (experiment 3), (Fig. 1). α-Methyl-D-mannopyranoside inhibited Con A binding to all metastatic variants at both temperatures (data for RT7-4b only shown).

TABLE IV. Rate of Attachment of ^{125}IUDR-Labeled Cells of the Parent Cell Line and the Metastatic Variants to Confluent Monolayers of Homotypic and Heterotypic Cells

	Percentage of cells adhering		
Cell line of the target monolayer	30 min	60 min	120 min
Monolayer of IAR6-7			
RT7-4b	34	49	57
RT7-4bA	20	52	59
RT7-4bE	15	54	70
RT7-4bL	34	74	81
RT7-4bM	43	72	86
Monolayer of RT7-4b			
RT7-4b	39	77	74
RT7-4bA	16	71	89
RT7-4bE	18	71	92
RT7-4bL	46	77	85
RT7-4bM	36	78	84
Monolayer of rat fibroblasts			
RT7-4b	60	64	77
RT7-4bA	45	77	84
RT7-4bE	64	84	97
RT7-4bL	66	82	87
RT7-4bM	67	83	83

TABLE V. Procoagulant Activities of the Metastatic Variants

		Procoagulant activity[a] observed with:			
Test sample	Plasma used	RT7-4bA	RT7-4bE	RT7-4bL	RT7-4bM
Conditioned serum-free medium	PPP[b]	152	8	71	44
	Factor VII-deficient PPP	26	19	34	20
Cell lysate	PPP	810	1,160	1,300	280
	Factor VII-deficient PPP	346	605	429	592

[a]Expressed in thromboplastin equivalence (TE) units: 1 TE = the activity of 1.5 μg rabbit brain thromboplastin.
[b]Platelet-poor plasma.

TABLE VI. Binding of ^{125}I-Con A to Monolayers of the Parent Cell Line and the Metastatic Variants

Cell line	^{125}I-Con A CPM ± SD[a]	Con A[b] (μg)
IAR6-7	6,186 ± 732	0.0155
IAR6-1-RT7	18,148 ± 2,455	0.0454
RT7-4b	30,841 ± 3,662	0.0771
RT7-4bA	11,710 ± 3,557	0.0293
RT7-4bE	502,770 ± 3,285	1.255
RT7-4bL	194,470 ± 3,862	0.486
RT7-4bM	33,495 ± 1,105	0.084

[a]Counts per minute of ^{125}I-Con A bound by 10^5 cells in 2 h at 37°C.
[b]Amount of ^{125}I-Con A binding to the cellular monolayers (μg per 10^5 cells).

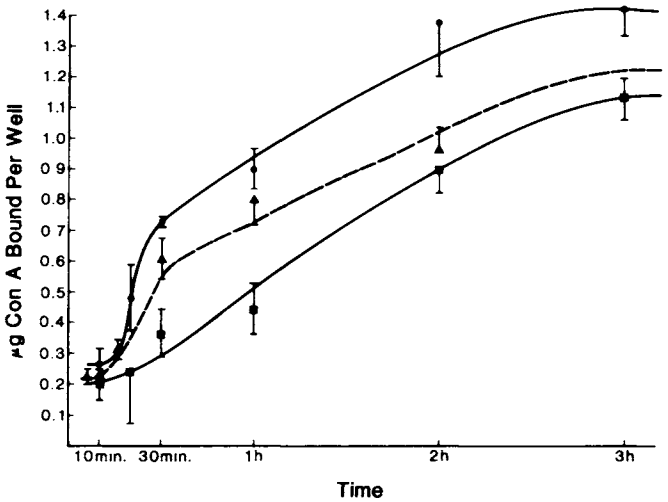

Fig. 1. Kinetics of Con A binding with time to monolayers of RT7-4b cells. ▲--▲ Con A binding at 4°C, ●—● Con A binding at 30°C, ■—■ Con A binding at 30°C in the presence of 0.7 mM α-methyl-D-mannopyranoside. Nonspecific Con A binding, which was unaffected by maximally inhibitory concentrations of α-methyl-D-mannopyranoside, was subtracted from all data points.

DISCUSSION

It is recognized that primary tumors are generally heterogeneous with respect to many cellular characteristics including metastatic ability. Metastatic variants isolated from such a tumor cell population are likely to be quite divergent in parameters unrelated to metastasis. To reduce this "background" variability, we isolated metastatic variants from a metastatically homogeneous, cloned tumor cell population after generation of metastatic heterogeneity during partial ascites adaptation. Chow and Greenberg [13] have recently confirmed that in vivo passage of a tumor line that maintains its homogeneity in vitro can lead to the generation of cellular heterogeneity. The mechanisms underlying in vivo generation of heterogeneity are not understood, but it is likely that they are intimately involved in tumor progression. Variants that represented the range of metastatic capabilities observed in our ascites-modified tumor cell population were chosen for study. Several in vitro properties of the metastatic variants were studied and correlated with the variants' metastatic capabilities in the lung colony assay (Table VII).

TABLE VII. Correlation of In Vitro Parameters With the Ability of the Metastatic Variants to Give Rise to Pulmonary Tumor Colonies After Intravenous Inoculation

In vitro parameter measured	Cell line in decreasing order of metastatic ability					Correlation with metastatic ability
	RT7-4bE	RT7-4bL	RT7-4b	RT7-4bM	RT7-4bA	
Population doubling time (h) (Table II)	12.8	13.6	12.9	10.2	11.1	---
Saturation density, cells per 21 sq cm growth area ($\times 10^5$) (Table II)	32.0 ± 2.5	28.0 ± 2.5	29.7 ± 6.3	29.3 ± 1.2	29.1 ± 6.6	---
Number of colony-forming cells shed into 1 ml of overlying medium in 48 h (Table II)	50 ± 23	232 ± 91	25 ± 3	20 ± 12	98 ± 8	---
Plasminogen activator production, % ^{125}I-fibrin release (Table III)	1.5	3.0	18.0	0.0	17.0	---
% Cells attached to IAR6-7 monolayers at 30 min (Table IV)	15	34	34	43	20	---
% Cells attached to RT7-4b monolayers at 30 min (Table IV)	18	46	39	36	16	---
% Cells attached to rat fibroblast monolayers at 30 min (Table IV)	64	66	60	67	45	---
Procoagulant production in conditioned medium, TE units for PPP/factor-VII deficiency. PPP (Table V)	81/19	71/34	---	44/20	152/26	–/–
Procoagulant activity in cell lysates, TE units, for PPP/factor-VII deficiency. PPP (Table V)	1,160/605	1,300/429	---	208/592	810/346	+/–
Con A (μg) bound by 10^5 cells in 2 h at 37°C (Table VI)	1.255	0.486	0.0771	0.084	0.0293	+++

Differences found between the population doubling times of the metastatic variants were small. However, the more metastatic variants exhibited slightly slower doubling times than the less metastatic ones. This agrees with some published reports [9, 10, 11], although the differentials in doubling times observed were not as great as those reported by others. This is probably to be expected, since the variants studied in this work were recently derived from a cloned parent cell line. In other reports [14, 15], no correlations could be found between growth rates in vivo or in vitro and experimental metastatic ability.

The circulation of cells within the vasculature of the host is a prerequisite to the formation of blood-borne metastases. However, the presence of circulating tumor cells does not necessarily imply that these are able to complete the metastatic process. Confluent cultures of the metastatic variants studied here shed viable cells into the culture medium. It has been suggested that the shedding of viable cells from confluent cultures might be related to the process of detachment of malignant cells from a primary tumor [12]. Large differences were observed between the number of cells shed from cultures of the four variant lines, but this did not correlate with the variants' metastatic phenotypes. The percentage of cells shed relative to the saturation density was low in all cases as compared with other tumor cell lines where this parameter has been studied [12]. Cell shedding in vitro, however, does not involve the complex tissue interactions that occur during tumor cell release in vivo. Such host-tumor interactions may include enzymatic release due to central necrosis of the tumor, inflammatory cell-mediated release of the tumor cells, or it may occur during the process of neoangiogenesis.

Tumor cell aggregation with other tumor cells or with cells of the immune system would allow the formation of larger tumor emboli increasing the efficiency of capillary trapping. Tumor cells that adhere well to endothelial cells should also be arrested better within the capillaries. It has been reported that highly metastatic B16 tumor cell lines adhere faster to both homotypic and heterotypic cells than less-metastatic B16 lines, with heterotypic adhesion faster than homotypic [16–19].

All of the tumor cell lines adhered faster to the heterotypic fibroblast cultures than to the parental cell lines. As with B16 variants, we find that heterotypic adhesion occurs faster than homotypic adhesion. Differences were found in attachment rates between the variants but these failed to correlate with their metastatic capabilities in the lung colony assay. While RT7-4bA exhibited slower heterotypic attachment than RT7-4bE in vitro, they exhibited similar patterns of arrest and trapping when they were injected intravenously [4]. Thus, it appears to be unlikely that the difference in heterotypic adhesion seen in this study was expressed in vivo so as to lead to differences in tumor cell arrest and trapping. Measurement of heterotypic adhesion of these variants to endothelial cell monolayers are in progress along with studies of the mechanisms of penetration of the monolayers. These may clarify the observations reported here.

It has been reported in several experimental systems that reduction or prevention of intravascular fibrin formation reduces tumor metastasis [20, 21]. However, other reports indicate that fibrin deposition plays no role in metastasis as it can be inhibited by various anticoagulants without effect on the final number of metastases found [22]. The importance of fibrin deposition to the trapping and arrest of tumor emboli probably varies quite widely between different tumors. Different tumor lines vary in their ability to aggregate platelets [18] and in their abilities to activate the clotting sequence (procoagulant activity).

Enhanced fibrinolysis due to plasminogen activator production might be expected to inhibit metastasis by reducing fibrin trapping of circulating tumor emboli. Plasminogen activator production might also serve to increase the invasiveness of tumor cells by aiding

the entry of tumor cells into the microvasculature and later their extravasation at secondary sites. Plasminogen activator levels for cell lines IAR6-7 and IAR6-1-RT7 have been previously reported by other workers [23]. The levels we obtained for IAR6-1-RT7 were close to those previously reported. The levels obtained for IAR6-7 were somewhat higher than those previously reported. This cell line had been in continuous culture in our laboratory for many months after receipt from Dr. Montesano when we first assayed its production of plasminogen activator. The high values obtained may thus be related to the relatively high spontaneous transformation frequency seen with this line after extensive propagation in vitro [3].

No correlation was seen between the metastatic ability of our tumor variants and their fibrinolytic activity. The parent cell line, RT7-4b, and the least metastatic variant, RT7-4bA, exhibited readily detectable levels of fibrinolytic activity. The other cell lines, including RT7-4bM, which has a metastatic phenotype similar to RT7-4b, exhibited little or nondetectable levels of fibrinolytic activity. The cell line IAR6-1-RT7 also exhibited a plasminogen-independent fibrinolytic activity. Because of the hepatic origin of these cells, it is possible that plasminogen-independent fibrinolysis was due to endogenous plasminogen production. Alternatively, a plasminogen-independent enzyme activity may be produced. A lack of correlation of plasminogen activator production with metastatic ability is also seen with B-16 metastatic variants [24]. It is unlikely from the data reported here and from previously reported data on the similar short-term fates of intravenously injected RT7-4bA and RT7-4bE variants [4] that fibrinolytic activity is important to the extravasation of these tumor emboli and other enzymes should be examined for this role.

Some tumor cell lines possess a marked ability to activate the clotting sequence. Such procoagulant activity may explain the enhanced intravascular formation of fibrin that is sometimes found with malignant disease [25]. A tumor-related procoagulant factor, cancer procoagulant A (CPA), has been extracted from human and animal tumors [26]. CPA directly activates factor X independent of factor VII to speed coagulation and thus differs from factor VII-dependent tissue thromboplastin [27]. We report procoagulant activities in thromboplastin equivalence units. When expressed in these units, tissue thromboplastin-like procoagulants would have similar activity for normal and factor VII-deficient plasmas, while CPA-like procoagulants would have a relatively higher procoagulant activity in factor-VII-deficient PPP. A CPA-like procoagulant activity was observed only for the variant RT7-4bM cell homogenate (Table V). The other variants exhibited lower levels of procoagulant activity in factor VII-deficient PPP than in normal PPP. This second procoagulant activity found was therefore not identical to the control tissue thromboplastin used.

The short-lived microthrombus that forms immediately after arrest of tumor cells in the microvasculature [28] could be initiated by host tissue trauma, the release of procoagulants by dead tumor cells, or by cellular secretion of procoagulants. In our system, no correlation was seen between the level of procoagulant activity secreted into serum-free medium and the metastatic phenotype. However, the procoagulant activities of cell lysates generally correlated with the metastatic phenotype. No correlation was noted between procoagulant activity and fibrinolytic activity, and they appear to be expressed independent of one another. This is in agreement with the results reported by others [29]. The levels of procoagulant activity found in vitro appear to have no correlation with the arrest and trapping of the variants RT7-4bE and RT7-4bA in vivo [4].

Generally, transformed cells exhibit enhanced agglutinability by low concentrations of lectins such as Con A or wheat germ agglutinin (WGA) [30]. When a population of metastatic tumor cells is treated with moderately toxic doses of WGA [31] or Con A binding cells are removed using immobilized Con A [32], tumor cells can be selected which have a

decreased ability to bind the selecting lectin. Such lectin-selected tumor cells are usually found to have altered metastatic phenotypes and in some cases to have lost their metastatic ability [31].

We found that the more metastatic variants bound more Con A per cell than did the less metastatic variants. The amount of bound ^{125}I-Con A could be affected by pinocytosis, capping, internalization, and receptor replacement, or other membrane phenomena. It could also be changed by the presence of binding sites with different kinetics of Con A binding. However, we found the kinetics of ^{125}I-con A binding to be the same for all variants studied both at 4°C and at 30°C. As was expected, monolayers of the untransformed parent cell line bound the least amount of ^{125}I-Con A. Con A has been shown in some experiments to bind slightly less well to the less metastatic B16-F1 variant than to the more metastatic B16-F10 [33]. In other experiments, no differences in Con A binding to these B16 variants were reported [16].

In earlier studies, we demonstrated that the cell surface morphology as seen by scanning electron microscopy correlates with the metastatic phenotype of these variants [4]. We find that cell surface morphology, Con A binding, and cell lysate procoagulant activity correlate with metastatic ability in the lung colony assay. The majority of the in vitro parameters examined do not correlate with this measure of metastatic ability. This general lack of correlation confirms results found with B16 and UV-2237 metastatic variants [14, 15]. Since many steps must be completed in sequence for tumor cells to successfully metastasize it may be that, as has been suggested by Fidler and Cifone [14], it is unrealistic to expect a single common cellular property conferring increased metastatic ability to exist for many cell types. The environment in vivo is so different from that in culture that in vitro assays on cultured tumor cells may not always be relevant to metastasis. Finally, it is possible that, for the variants examined here, immune parameters are of relatively greater importance in determining differences in the largely quantitative lung colony assay for metastatic ability.

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