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Spontaneous Fusion Between Metastatic Mammary Tumor Subpopulations

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This study describes a differential frequency of spontaneous fusion between metastatic and nonmetastatic subpopulations derived from a single mouse mammary tumor. Subpopulations 66, 66cl4 (a variant of 66 which is resistant to both thioguanine and ouabain), 410.4, and 44FTO (a thioguanine-resistant, ouabain-resistant derivative of 410.4) spontaneously metastasize from subcutaneous and mammary fatpad sites. Subpopulations 168, 168FARO (a diaminopurine-resistant, ouabain-resistant derivative of 168), 67, 68H, and 410 do not. The ability of these subpopulation lines to fuse spontaneously in vitro was determined after coculturing a drug-resistant line with a wild-type line in nonselective media. After 16–20 h of coculture, cells were plated in the appropriate media to select for fusion products—either HAT (hypoxanthine, aminopterin, thymidine) plus ouabain or AA (alanosine, adenine) plus ouabain—to determine the number of colony-forming cells (fusion products) present per 10^4 cells plated. When both subpopulations of the pair in the fusion mixture were metastatic, a significantly greater number of fusion products was recovered than if one or both of the subpopulations in the fusion mixture was nonmetastatic, with one exception: line 410 readily fused with both 66c14 and 44FTO. Subline 410 was highly metastatic when originally isolated but lost its metastatic competence after a brief time in tissue culture.

Key words: hybrid cells, metastasis, heterogeneity, generation of aneuploidy

Cells from metastatic lesions are frequently more aneuploid than cells from primary tumors [1–4]. The mechanisms by which cells become aneuploid are unclear, but such cells could result from endoreduplication or by cell fusion, which might then be followed by asymmetric segregation. Cell fusion could play a significant role in progression by allowing the rapid assimilation of multiple properties from various subpopulations rather than requiring a single cell lineage to undergo sequential mutagenic alterations. Furthermore, asymmetric segregation subsequent to cell fusion offers a mechanism by which heterogeneity might be rapidly generated.

In many instances hybrids of nonmetastatic lymphatic tumor cells and normal host cells, usually macrophages or lymphocytes, have been found to be metastatic [5–

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9]. It is not perfectly clear from those studies whether the fusion of tumor cell with one of these normal cells results in a more metastatic cell or if a rare metastatic subset of tumor cells is more likely to fuse. In experiments designed to study growth interactions between normal mammary gland cultures and metastatic mammary tumor cells in vitro, we were surprised to find that a significant number of hybrid cells formed. The purpose of the present investigation was to determine, utilizing subpopulations of a mouse mammary carcinoma, the extent to which these carcinoma cells spontaneously fuse and whether metastatic subpopulations are more likely to fuse than are nonmetastatic subpopulations.

MATERIALS AND METHODS

Cells

Tumor cell lines 66, 67, 168, 410, and 410.4 were isolated from a single, spontaneously arising mammary tumor from a BALB/cfC_3H mouse [10,11]. The thioguanine-resistant, ouabain-resistant cell lines 66c14 and 44FTO were isolated from lines 66 and 410.4, respectively, as previously described [12]. The 2,6-diaminopurine (DAP), ouabain-resistant cell line 168FARO was derived from line 168 by sequential selection in increasing concentrations of DAP until resistance had increased from 2 μg/ml to 100 μg/ml, followed by selection for resistance to 3 mM ouabain. When transplanted to subcutaneous sites in syngeneic mice, all of these cell lines form tumors but only lines 66, 66c14, 410.4, and 44FTO regularly form metastases [13–15]. Lines 67, 168, and 168FARO very rarely metastasize from the subcutis. Line 410 was originally isolated from a lung metastasis but lost metastatic potency after a brief time in tissue culture [13]. Occasionally, metastases do form in the lungs of a mouse bearing tumor 410. All lines were found to be free of mycoplasma contamination by the Hoechst staining method [16] and were free of mouse pathogenic viruses (PVM, Reo3, Sendai, GDVII, K, Polyoma, MVM, MAD, MHV, LCM, and Ectromelia) as determined by MAP tests performed by Microbiological Associates (Bethesda, MD).

Media and Drugs

The tumor cell lines 168, 410, and 410.4 were maintained in monolayer culture at 37°C in 5%- CO_2 in air atmosphere in Waymouth's Medium MB752/1 supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. Lines 66, 66c14, 168FARO, and 44FTO were maintained in monolayer culture at 37°C in 10% CO_2 in air atmosphere in Dulbecco's Modified Eagle medium supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), mixed nonessential amino acids (1 mM), and 10% fetal bovine serum (DME-10).

Adenine, ouabain, 6-thioguanine, 2,6-diaminopurine, and a concentrated HAT mixture were purchased from Sigma Chemical Co. (St. Louis, MO). Alanosine was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). The final concentrations of selective drugs in DME-10 used were as follows: for HAT medium, hypoxanthine, 100 μM, aminopterin, 0.4 μM, thymidine, 16 μM; for thioguanine medium, 60 μM; for ouabain, 3 mM; for DAP, 250 μM; and for AA medium, alanosine, 50 μM, adenine, 50 μM.

Fusion In Vitro

To determine the ability of cell lines to spontaneously form cell hybrids, 5×10^5 cells of a cell line with drug-resistant phenotypes (66c14, 44FTO, or 168FARO) were mixed with 5×10^5 cells of a nonresistant wild-type line in DME-10 in a T-25 flask. After 16–20 h at 37°C in 10% CO_2 in air atmosphere, the cells were suspended with a solution of trypsin and EDTA. A single-cell suspension was formed by vigorously pipetting the cells and then passing the cell suspension two or three times through a 25-gauge needle. The cells were counted by hemacytometer and plated at 10^4 cells per 600-mm dish in DME-10 media containing selective drugs permissive only to the growth of fusion products. Medium containing HAT plus ouabain was used to select for fusion products between $HGPRT^-$(deficient in the enzyme hypoxanthine, guanine-phosphoribosyltransferase), ouabain-resistant cells and $HGPRT^+$, ouabain-sensitive cells. Medium containing AA plus ouabain was used to select for fusion products between $APRT^-$(deficient in the enzyme adenine phosphoribosyltransferase), ouabain-resistant cells and $APRT^+$, ouabain-sensitive cells [17, 18]. After 10 days incubation at 37°C in 10% CO_2 in air atmosphere, resulting colonies (>32 cells) were counted after fixing with Carnoy's solution and staining with crystal violet [19].

Fusion In Vivo

Cells from monolayer culture were injected into syngeneic BALB/c mice in a volume of 0.1 ml. Tumors were removed when they reached a mean diameter of 10 mm. Cell suspensions were made from the tumors using collagenase type III, hyaluronidase, and protease type IX as previously described [15] and plated in selective media to determine the cellular composition of the tumors.

Analysis of DNA Content

Cells were fixed and stained with propidium iodide as previously described [19] and analyzed for DNA content with a Becton-Dickenson FACS 440 flow cytometer.

RESULTS

The ability of the subpopulations to form colonies in various selective media is given in Table I. Only 66c14 and 44FTO grew in media containing thioguanine and ouabain and failed to grow in HAT. Only 168FARO grew in media containing both DAP and ouabain; 168FAR and 168FARO were uniquely unable to grow in AA. None of the subpopulations was able to grow in HAT plus ouabain or in AA plus ouabain.

To test the tendency of two nonmetastatic cell lines to fuse spontaneously in vitro we cocultured 168FARO with line 67, 168, or 410. Few fusion products were subsequently recovered by incubating the cell mixtures in AA plus ouabain (Table II). Typically, less than one colony-forming hybrid cell could be detected per 10^4 cells in the fusion mixtures.

If one cell line in the fusion mixture was metastatic, either 66c14 or 44FTO, and the other cell line was nonmetastatic, fusion was still a rare event. Typical experimental data are given in Table III. Lines 67, 168, and 168FAR did not readily fuse with either 66c14 or 44FTO as determined by the recovery of colonies growing in HAT plus ouabain. However, line 410, which is only sporadically metastatic,

TABLE I. Growth of Mouse Mammary Tumor Cell Lines in Selective Media

Cell line	Frequency of survival[a] in					
	HAT	T+O	HAT+O	AA	DAP+O	AA+O
66	.48	$<2 \times 10^{-5}$[b]	$<2 \times 10^{-5}$	.41	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
66c14	$<2 \times 10^{-5}$	.40	$<2 \times 10^{-5}$	.36	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
410.4	.23	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	.25	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
44FTO	$<2 \times 10^{-5}$	.32	$<2 \times 10^{-5}$	.29	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
410	.29	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	.34	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
67	.12	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	.16	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
168	.41	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	.38	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
168FAR	.40	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$
168FARO	.37	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	$<2 \times 10^{-5}$	.60	$<2 \times 10^{-5}$

[a]Frequency of survival $= \frac{\text{total number of colonies}}{\text{total number of cells plated}}$.

[b]Five dishes were plated with 1×10^4 cells each and no colonies grew; thus the frequency of survival is unknown but less than 2×10^{-5}.

TABLE II. Colony Formation in Selective Media by Fusion Mixtures of Paired Nonmetastatic Cell Lines

Cell lines in fusion mixture	Frequency of survival in		
	AA	DAP+O	AA+O
168FARO + 67	.27	.21	4×10^{-5}
168FARO + 168	.20	.11	5×10^{-5}
168FARO + 410	.24	.21	9×10^{-5}

TABLE III. Colony Formation in Selective Media by Fusion Mixtures of Paired Metastatic and Nonmetastatic Cell Lines

Cell lines in fusion mixture	Frequency of survival in		
	HAT	T+O	HAT+O
66c14 + 67	.18	.17	1.2×10^{-4}
66c14 + 168	.21	.14	$<2 \times 10^{-5}$
66c14 + 168FAR	.17	.27	1.3×10^{-5}
44FTO + 67	.23	.21	7×10^{-5}
44FTO + 168	.23	.22	1.8×10^{-4}
44FTO + 168FAR	.23	.26	5×10^{-5}
66c14 + 410	.19	.25	3.3×10^{-3}
44FTO + 410	.22	.25	5.5×10^{-3}

readily fused as evidenced by the recovery of 55 and 33 colony-forming cells per 10^4 cells of the fusion mixtures with 44FTO and 66c14, respectively. The ability of 410 to fuse was equal to that of the metastatic lines.

If both cell lines in the fusion mixture were metastatic, fusion products were readily obtained (Table IV). Both wild-type and drug-resistant forms of the metastatic subpopulations 66 and 410.4 were matched in each possible pairing with equivalent results. Data from all 39 experiments are shown in Figure 1. When both lines of the pair in the fusion mixture were metastatic, a significantly greater ($P < .002$) number of hybrids was recovered (median of 18 per 10^4 cells for 18 experiments) than if one of the lines in the fusion mixture was nonmetastatic (median of 0.6 per 10^4 cells for 15 experiments).

TABLE IV. Colony Formation in Selective Media by Fusion Mixtures of Paired Metastatic Cell Lines

Cell lines in fusion mixture	Frequency of survival in HAT	T+O	HAT+O
66 + 66c14	.19	.14	8.2×10^{-3}
66c14 + 410.4	.30	.24	7.1×10^{-3}
66 + 44FTO	.17	.23	1.3×10^{-3}
410.4 + 44FTO	.33	.23	5.6×10^{-3}

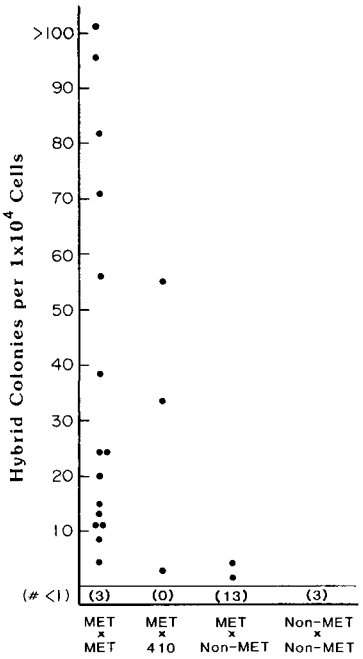

Fig. 1. Fusion frequency between paired tumor subpopulations. Each symbol represents the mean of five replicate wells for an individual experiment. The numbers of experiments in which the mean of five replicates indicated that fewer than one hybrid cell existed per 1×10^4 cells plated from a fusion mixture is also given.

Of 16 clones selected with HAT plus ouabain medium, 15 have displayed the DNA content expected by summing the DNA content of the parental cell lines in the fusion mixture. Figure 2 shows the DNA histograms for the parental lines and a hybrid clone from one such experiment. The one exception arose from a mixture of 66c14 and 66; this 2n clone was presumably either a revertant $HGPRT^-$, ouabain-resistant cell to $HGPRT^+$, a spontaneous mutation of $HGPRT^+$, ouabain-sensitive cell to ouabain resistant, or a spontaneous mutation of a $HGPRT^-$, ouabain-resistant cell to an increased resistance to aminopterin.

Colonies were not formed in HAT plus ouabain by cells from dissociated tumors arising in mice after SC injection of either 66c14 or 66 alone. However, tumors consisting of a mixture of 66 and 66c14 contained a significant number of hybrid cells. The data of Table V indicate that 98% of the clonogenic cells recovered from a tumor resulting from the injection of 66c14 cells grew in media selective for 66c14.

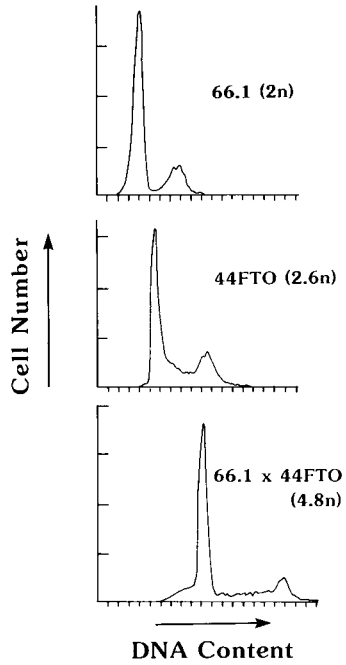

Fig. 2. Verification of the hybrid nature of a selected clone. The DNA histograms for the parental cells in the fusion mixture (66.1 and 44FTO) and the cell selected by growth in HAT plus ouabain (66.1 × 44FTO) are illustrated. Vertical and horizontal scales are identical for the three histograms.

TABLE V. In Situ Fusion: Colony Formation in Selective Media by Cells From Dissociated Tumors

Cells injected to form tumors	Percent of recovered cells which grew in[a]		
	HAT	T+O	HAT+O
1×10^5 line 66c14	2 (0–4)	98 (96–100)	0
1×10^5 line 66	100	0	0
2×10^4 66 + 1.8×10^5 66c14	71 (68–79)	21 (16–25)	8 (4–10)

[a]Six replicates were plated in each media for each tumor. Numbers given are the medians of the mean of the six replicates for five individual tumors. Figures in parenthesis represent the range of the means.

An additional 2% which grew in HAT were most likely host cells (fibroblasts?) rather than fusion products, because no colonies formed in HAT plus ouabain. Cells from dissociated tumors resulting from SC injection of line 66 cells grew only in HAT as expected. This indicates only that the rate of spontaneous mutation to ouabain resistance was too low to be detected. However, tumors formed by a mixture of 66 and 66c14, with 66c14 in excess to compensate for the tendency of 66 to overgrow 66c14 in mixtures, consisted of 8% (median of five tumors) hybrid cells.

DISCUSSION

The frequency with which the mammary tumor subpopulations fuse with one another is much higher than that described for various other cell types. Even the

nonmetastatic subpopulations spontaneously form hybrid cells at a frequency of about 5×10^{-5}, which is greater than the range reported in the literature for other cell types: for example, 1×10^{-5} for spontaneous hybrid formation between mouse CI-Id cells and human WI-18Va2 cells [20], 4×10^{-6} between 3T3 cells and Chinese hamster cells [21], and less than 2×10^{-7} between mouse leukemia cells or mouse leukemia and Chinese hamster fibroblasts [22]. The frequency with which the metastatic subpopulations fuse (a median frequency of 2×10^{-3} with 1×10^{-2} being observed in one experiment) exceeds those reported for induced fusion by Sendai virus (5×10^{-5}, ref. [20]) or polyethylene glycol (2×10^{-4}, ref. [21] 3×10^{-4} to 4×10^{-5}, ref. [22]). The subpopulations were all derived from a BALB/cfC_3H mammary tumor and express the mouse mammary tumor virus (MMTV) to varying degrees [23]. MMTV has been reported to increase the frequency of hybridization [24] much like other enveloped viruses. However, the effect was seen with a cell line derived from mink lung but not with a mouse mammary tumor line or a line derived from normal mouse mammary gland [24]. Furthermore, the increase in spontaneous fusion of the mink lung cells was on the same order of magnitude as Sendai and Semliki Forest virus and was only seen at pH below 6. Therefore, it is unlikely that the frequency of hybridization seen with our cell lines can be attributed to MMTV infection. Roos and Choppin [25] found that mouse L fibroblast cells selected for resistance to PEG-induced fusion were more malignant, more metastatic, than the original L cells which readily formed hybrids following PEG treatment. The ability to fuse correlated with the lipid content of the L cell variants. We have not, as yet, determined the lipid content of our cell lines. The apparent discrepancy between our results, in which metastatic cells spontaneously fuse more readily, and the results of Roos and Choppin, in which metastatic cells fuse less frequently in response to PEG, might be due to a difference in cell systems or be because different mechanisms are responsible for spontaneous and PEG-induced fusion. Indeed, metastatic L cells concurrently demonstrated resistance to PEG-induced fusion and increased fusion rates induced by enveloped viruses [25].

A membrane property which might be important in fusion, and itself might be dependent upon lipid composition, is membrane fluidity. It has been reported that metastatic cell membranes are more fluid, or easily deformed, than membranes of nonmetastatic cells [26, 27].

Cells of our metastatic mammary tumor subpopulations may share a unique adhesion molecule [28] which allows them to adhere and fuse. Although expression of normal cell adhesion molecules may be reduced in cancer cells [29], Raz et al [30] have reported that the expression of a cell-surface lectin important in cell aggregation correlates with transformation and the acquisition of the metastatic phenotype.

Regardless of the mechanistic explanation, because the metastatic cells clearly fused spontaneously in situ, an important consequence of spontaneous fusion of tumor subpopulations is the potential for metastatic cells to rapidly acquire population characteristics such as resistance to chemotherapy. The occurrence of 8% hybrid cells in a metastatic tumor is not unprecedented; Hart described a similar frequency in B16-F10 tumors [31]. It remains to be seen whether the hybrid tumor-tumor cells are stable or rapidly generate heterogenous subpopulations. Chromosome loss is very rapid after the fusion of Eb tumor cells with normal host cells [9], but that probably reflects, in part, the necessity for loss of the tumor suppressor genes from the normal cells [32–35]. Our initial experiences with the tumor-tumor hybrids indicate that they

are relatively stable, as assessed by total DNA content. However, unlike a tumor-normal cell hybrid, there is no strong selective pressure elicited against the tumor-tumor hybrid cells. Future experiments may elucidate the impact of spontaneous fusion on progression and the generation of heterogeneity.

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