Production of a More Aggressive Tumor Cell Variant by Spontaneous Fusion of Two Mouse Tumor Subpopulations

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ABSTRACT

A hybrid cell formed by the spontaneous fusion of two sister subpopulations was isolated and was found to combine clinically aggressive features of both parental cells. Subpopulation 168FAR is highly tumorigenic but does not metastasize spontaneously from a s.c. site. Subpopulation 44FTO is a variant selected for resistance to 6-thioguanine and to ouabain. It is poorly tumorigenic but spontaneously metastasizes to the lungs and livers of mice in which primary tumors do form. The two parental subpopulations are differentially sensitive to chemotherapeutic drugs; 168FAR is relatively more resistant to methotrexate, whereas 44FTO is more resistant to 5-fluorouracil. Both are about equally sensitive to melphalan. The hybrid 168FAR × 44FTO clone was isolated by growth in hypoxanthine-aminopterin-thymidine medium which also contained 3 mmouabain; neither 168FAR nor 44FTO survive in this medium. The hybrid nature of the isolated clone was confirmed by cytogenetic analysis and determination of DNA content. The hybrid clone was highly tumorigenic, spontaneously metastasized from the subcutis, was nearly as resistant to 5-fluorouracil as the most resistant parental line, and was more resistant to both melphalan and methotrexate than either parental subpopulation. These results illustrate the role that cell fusion could play in progression by allowing the rapid assimilation of aggressive phenotypes from distinct coexisting subpopulations.

INTRODUCTION

Numerous studies have found that increasing DNA content correlates with malignant behavior (1–7). Patients with ovarian cancer whose tumors were diploid survived significantly longer than patients with aneuploid tumors in a study by Friedlander et al. (2). Most of the aneuploid tumors had DNA indices in the range of 1.5 to 2. Frankfurt et al. (3) found that aneuploid human prostate cancers were much more likely to be invasive and metastatic than diploid tumors. Survival of colon carcinoma patients was reported by Wolley et al. (4) to be much better for patients with diploid tumors than ones with aneuploid tumors. For human breast cancer, Auer et al. (5) found that patients with scattered DNA values outside the normal range had the worst prognosis, and Coulson et al. (6) found that patients with hypertetraploid and multiploid DNA histograms had worse prognoses than patients with diploid and hyperdiploid tumors. However, patients with hypodiploid tumors also had a poor prognosis in the latter study, and other studies of breast cancer patients have not revealed any correlation between DNA content and survival (8). Cause and effect relationships between the acquisition of malignant properties and increased DNA are not established. Curiously, near-tetraploid tumors may not be more aggressive than pseudodiploid tumors (3). The appearance of aneuploid cells may mirror a more general defect in the control of the cell cycle. Increased cellular DNA may result from partial or complete endoreduplication, asymmetric segregation, gene amplification, or the fusion of two cells. The role that gene amplification may play in progression to a therapy-resistant form has been well documented (9, 10). The observed gene amplification with repeated selection for resistance to increasing concentrations of drugs, such as methotrexate, is typically in the form of double minutes or homogeneous staining regions. Goldberg et al. (11) suggested that fusion of cancer and host cells could result in tumor progression, and it has been reported that the fusion of tumor cells with normal host blood cells results in a metastatic hybrid (12–14). Because tumor subpopulations fuse with each other in situ (15, 16), we have explored the possibility that the spontaneous formation of hybrids between different subpopulations might lead to a more aggressive variant combining the most malignant features of each parental cell.

MATERIALS AND METHODS

Mice. Female BALB/c mice, 7 to 9 weeks old, were used. These mice were produced at the Michigan Cancer Foundation by a BALB/c breeding colony that was established by cesarean derivation of a litter of mice from BALB/cF3H parents that were obtained from the Cancer Research Laboratory (Berkeley, CA).

Cell Lines. The cell lines 168FAR and 44FTO are drug-resistant variants of two sister subpopulations, 168 and 410.4, which were isolated from a single, spontaneously arising, mammary tumor of a BALB/cF3H mouse (17, 18). The DAP-resistant cell line 168FAR was derived from line 168 by sequential selection in increasing concentrations of DAP until resistance had increased from 5 to 250 µM. The thioguanine-resistant (60 µM)ouabain-resistant (3 HIM) line 44FTO was isolated from 410.4 as previously described (19). Both cell lines were certified Mycoplasma free, using DNA fluorochrome stain plus UV microscopy, by Bionique Laboratories (Saranac Lake, NY).

Media. Cells were routinely maintained in DME-10 medium that consisted of DME supplemented with 5% iron-supplemented calf serum (low endotoxin serum; Hyclone Sterile Systems, Inc., Logan, UT), 5% fetal bovine serum (low endotoxin serum; GibCO, Grand Island, NY), 1 mm mixed nonessential amino acids, and 2 mm glutamine.

Drugs. The selective drugs ouabain, 6-thioguanine, DAP, and a concentrated HAT mixture were purchased from Sigma Chemical Co. (St. Louis, MO). The final concentrations of selective drugs, in DME-10, used were as follows: for HAT, hypoxanthine, 100 µM, aminopterin, 0.4 µM, and thymidine, 16 µM; for thioguanine, 60 µM; for DAP, 250 µM. The therapeutic drugs used to determine the relative levels of innate resistance of the cell lines in vitro were melphalan (Sigma), me-thotrexa-te (Lederle Laboratorys, Pearl River, NY), and 5-fluorouracil (Solu-pak, Franklin Park, IL).

Cells were removed from monolayer cultures with trypsin:EDTA, rinsed with DME-10, passed through a syringe with a 25-gauge needle several times as necessary to obtain a single-cell suspension, and plated at low density in 6-well tissue culture cluster plates (200 cells/well) in DME-10 that contained the cytotoxic drug. Plates were incubated for 7 to 11 days at 37°C in a 10% CO2 in air atmosphere, fixed with Carnoy’s solution (methanol:acetic acid, 2:1), and stained with crystal
violet. Colonies of greater than 32 cells were counted with a dissecting microscope.

Tumors. Cells from monolayer culture were suspended in Hanks' balanced salt solution and injected s.c. into mice in a volume of 0.1 ml. Tumors were measured twice a week in two perpendicular dimensions with Vernier calipers. Tumor size in mm\(^3\) was calculated by the formula 
\[ a \times b^2 + 2, \]
where \( b \) is the smaller of the two diameters. For each individual tumor, the volume between approximately 0.1 and 0.5 cm\(^3\) was fitted to an exponential growth curve, using linear regression analysis of the logarithm transformation of tumor volume, and the tumor doubling time was obtained from each fitted curve. Animals were sacrificed by cervical dislocation when they became moribund and necropsies were performed to enumerate grossly visible metastases, primarily in the lungs and livers.

Spontaneous Fusion. To obtain a hybrid cell line, 5 \times 10\(^5\) cells of line 44FTO were mixed with 5 \times 10\(^5\) cells of line 168FAR in a T-25 flask. After incubation for 20 h at 37°C in a 10% CO\(_2\) in air atmosphere, the cells were suspended with trypsin and EDTA, counted by hemacytometer, and plated at 1 \times 10\(^6\) cells/60-mm dish in DME-10 that contained HAT and ouabain, to select for fusion products. These two lines were previously found to fuse at a frequency of 5 \times 10\(^{-5}\). A similar frequency was observed in this experiment. To ensure that a single hybrid was obtained, subclones of one colony were derived by dilution cloning in 96-well plates. One clone, which appeared to have the shortest doubling time, was further analyzed.

Analysis of DNA Content. Cells were fixed and stained with propidium iodide as described previously (20) and were analyzed for DNA content with a Becton-Dickinson FACStar flow cytometer.

Cytogenetic Analysis. Confluent cultures were split 1:5 and harvested 24 h later. Cell cultures were exposed to Colcemid (0.05 µg/ml) for 1 h before harvesting. The cells were swelled in 0.075 M potassium chloride for 30 min and then fixed in 1:3 methanol:glacial acetic acid. The fixative was changed 3 or 4 times and cell suspensions were dropped onto cold wet slides. Chromosome preparations were incubated for 10–14 days at 40°C and then stained by GTG banding (21, 22). For each culture, 30–50 metaphases were analyzed.

RESULTS

DNA Content of Parental Subpopulations and the Hybrid. Neither 168FAR nor 44FTO grow in medium that contains both HAT and ouabain (16). Fewer than 1 in 2 \times 10\(^6\) cells, i.e., no colonies from five replicate wells each containing 1 \times 10\(^6\) cells, were able to survive. The hybrid 168FAR \times 44FTO clone had a colony-forming efficiency of 67% in medium containing HAT plus ouabain. The hybrid was unable to grow in either DAP- or thioguanine-containing medium (<2 \times 10\(^{-5}\) colony-forming efficiency). As depicted in Fig. 1, the DNA content of the hybrid approximated the sum of the DNA of the two parental subpopulations. In different trials, the DNA indices ranged from 1.55 to 1.58 for 168FAR, 1.30 to 1.38 for 44FTO, and 2.71 to 2.85 for the hybrid.

Cytogenetic Analysis. The 168FAR line has a stable karyotype with a chromosome number ranging from 57 to 63. All cells carry two copies of a large metacentric marker chromosome (M1) and one copy of a medium-sized metacentric marker (M2). These marker chromosomes are depicted in Fig. 2.

The chromosome number of 44FTO ranges from 48 to 53, with a mode of 49. In most 44FTO cells, all the chromosomes are telocentric. However, 30% of the 44FTO cells display a large metacentric marker [M3 (Fig. 2)], which appears to be an isochromosome of chromosome 4.

Cells derived from the hybrid clone showed chromosome numbers ranging from 93 to 107, indicating that they contain nearly all the chromosomes from both parents. A typical metaphase (Fig. 3) contains two copies of M1 and one copy of M2. M3 was found in about 60% of the cells. Additional new stable and unstable chromosome aberrations were also present, including a small number of double minutes in many cells. The new stable recombinant chromosomes were seen in 90% or more of the cells. Unstable recombinants such as multcentrics or rings were seen in a small proportion of cells.

Fig. 1. DNA content by cytofluorographic analysis. The DNA histograms for the parental cells in the fusion mixture (168FAR and 44FTO) and the cell selected by growth in HAT plus ouabain (168FAR \times 44FTO) are illustrated. DNA index, modal DNA content of the indicated cell divided by the model DNA content of a normal diploid mouse cell.

Fig. 2. Marker chromosomes from parental mammary tumor cell lines 168FAR (M1 and M2) and 44FTO (M3).
Fig. 3. G-banded metaphase of a hybrid 168FAR × 44FTO cell showing the presence of the M1, M2, and M3 chromosomes.

Fig. 4. Growth of tumor lines in the subcutis. Cells (1 × 10^6) of line 168FAR (A), 44FTO (B), or the hybrid 168FAR × 44FTO (C) were injected s.c. in the inguinal region of 10 mice each. Mice were examined twice weekly and tumors were measured in two perpendicular diameters. Volumes were calculated using the formula \(a \cdot \frac{b^2}{2}\), where \(b\) was the smaller of the two diameters and \(a\) the larger. The growth of tumor in one animal is indicated by each line. Tumors were not detectable in 4 mice that were treated with 44FTO, 70 days after injection.
bearing 168FAR. The hybrid cells were of intermediate metastatic potential. Of 19 mice that were necropsied, 7 had metastatic nodules in their lungs (37% incidence). Although one might expect metastasis to become more likely with time and with increasing primary tumor size, Fig. 5 shows that the differential capacity to metastasize was not simply due to time and size differences. 44FTO metastases were evident whether animals had been bearing primary tumors for 32 or 105 days and 168FAR did not metastasize whether primary tumors had grown for 39 or 105 days. None of the mice bearing the hybrid line tumor were necropsied later than 55 days after s.c. implantation, but metastases were evident as early as 40 days. None of nine animals with 168FAR tumors exceeding volumes of 10 cm³ at necropsy had detectable metastases; none of the 44FTO tumors exceeded 3 cm³ at necropsy. The range of volumes of hybrid-type tumors in which the necropsies revealed the presence of metastases was 3.7 to 12.2 cm³.

Relative Sensitivity to Chemotherapeutic Agents of Parental Subpopulations and the Hybrid. As indicated by the data in Table 2, the hybrid cell line was at least as resistant to each of the three chemotherapeutic drugs tested as was the most resistant parental subpopulation. In three individual experiments, subpopulation 44FTO was consistently the most sensitive to methotrexate; 168FAR was less sensitive and the hybrid was consistently the least sensitive cell line. Fig. 6 illustrates the results of a typical experiment.

Fig. 7 illustrates one of five experiments assessing the relative sensitivity of the cells to melphalan. In all but one experiment, 168FAR was somewhat more sensitive to melphalan than 44FTO. The hybrid was consistently less sensitive than either of the parental lines.

Subpopulation 168FAR was more sensitive to 5-fluorouracil (three experiments) than either 44FTO or the hybrid. The sensitivities of 44FTO and the hybrid were similar. Fig. 8 illustrates one experiment in which the hybrid was slightly more resistant than 44FTO but, in two other experiments, 44FTO was slightly more resistant than the hybrid.

**DISCUSSION**

When Goldenberg et al. (11) found that human tumor cells transplanted into hamster cheek pouches fused with hamster cells to form a more aggressive hybrid, *i.e.*, better able to grow in the xenogenic host, they suggested that a similar mechanism could lead to progression in the autochthonous situation. They envisioned a cancer cell-normal cell hybrid which would more weakly express tumor-specific transplantation antigens, resulting in a more aggressive tumor. We now know that tumor cell-normal cell hybrids are likely to be less tumorigenic than the parental tumor cell, due to tumor suppressor genes (23–25). However, with time, asymmetric segregation may result in a cell which has lost the suppressor genes and is able to again express the tumorigenic phenotype (23, 26, 27). Such post-
FUSION-FUELED PROGRESSION

Several investigators have reported that the fusion of a tumor cell with a normal host cell may result in the progression from a nonmetastatic to a metastatic phenotype (12-14). In these examples, both the tumor cells and normal host cells involved in the fusion were of a lymphoreticular origin. Hybrids between S-194 cells (a plasmacytoma) and normal spleen cells were found to be much less stable than hybrids between the plasmacytoma and mouse fibroblasts, in a study by Oikawa et al. (28). Lagarde (29) reported that hybrids of MDAY-D2 (a lymphoreticular tumor) and splenocytes were tumorigenic and metastatic (as was the parental tumor), whereas hybrids of MDAY-D2 and primary lung fibroblasts senesced in vitro after six or seven population doublings. The tumor-splenocyte hybrids in Lagarde's study (29) were not as unstable as those reported by Oikawa et al. (28). Cowell and Franks (30) found that fusion with bladder epithelium but not bladder mesenchymal cells suppressed the tumorigenicity of bladder carcinoma cells in the mouse. Thus, the fate of the hybrid (i.e., growth/senescence, tumorigenic/nontumorigenic, metastatic/nonmetastatic, stable/unstable karyotype) is dependent upon the tumor cell and upon the host cell type participating in the fusion.

Independent tumor subpopulations may also fuse spontaneously in vivo. We found that nearly 10% (median of 8%, range of 4-10%) of clonogenic cells from dissociated tumors arising after injection of a mixture of two subpopulations were hybrids (16). This was determined by injecting a mixture of a "universal fuser" (31) subpopulation, which was hypoxanthine-guanine phosphoribosyltransferase-deficient, ouabain-resistant, and a wild-type subpopulation. Colonies of hybrids were quantitated by colony formation in media containing HAT and ouabain. A hybrid formed by the fusion of the universal fuser tumor cell with a normal host cell would also form colonies in medium containing HAT and ouabain but no such colonies were detected from dissociated tumors formed solely from the hypoxanthine-guanine phosphoribosyltransferase-deficient, ouabain-resistant tumor cells (16). Presence of the wild-type tumor subpopulations was required for the formation of hybrids. Hart (15) reported a similar content of tumor subpopulation hybrids in B16 melanomas.

In the present study, the hybrids were formed in vitro using tumor subpopulations which had been passed in vitro to ensure that a host cell was not one of the fusion partners. These hybrids were created to demonstrate that the fusion of different coexisting tumor subpopulations can assimilate clinically aggressive features of each into a single new tumor variant. This provides a mechanism by which tumors could very rapidly progress. If asymmetrical segregation occurs, heterogeneity may also be created through this process. In fact, one might expect that tumors would be much more heterogeneous in DNA content than has been reported. In studies of human cancer, only 4% of prostate cancers (6), 4% of colorectal adenomas (7), 9% of breast cancers (4), and 16% of ovarian cancers (5) were found to be multiploid, i.e., to have more than one aneuploid peak, by fluorescence-activated cell sorter analysis. In most of these cases, only two peaks were evident. Perhaps clonal dominance, as described by Miller et al. (32, 33), by Kerbel et al. (34), and by Waghorne et al. (35), masks the presence of multiple minor subpopulations which, however, can become major components under selective conditions or if the dominant population is removed (e.g., by therapy or surgery).

Cifone and Fidler (36) have suggested that metastatic cells have a higher rate of spontaneous mutation, which could enable them to respond to selective forces. Others have not found metastatic cells to be more genetically unstable (37, 38). We have previously reported that, in our mouse mammary model, the rate of spontaneous fusion is very high between two metastatic subpopulations; fusion occurs at lower frequency if one fusion generation cells need not have lost all normal chromosomes/genes, however, and thus a tumor cell with new characteristics may eventually result. Such cells may or may not display more aggressive features than the original tumor cell which existed before fusion. Several investigators have reported that the fusion of a tumor cell with a normal host cell may result in the progression from a nonmetastatic to a metastatic phenotype (12-14). In these examples, both the tumor cells and
of the tumor subpopulations is nonmetastatic (16). Metastatic subpopulations might thus be more able to evolve under selective pressures than nonmetastatic cells, by a method other than mutation. However, we have found that our metastatic lines do not fuse at high frequency with metastatic variants of other tumors.4 It is not surprising that the mechanisms by which progression occurs and heterogeneity is generated are heterogeneous, differing for different tumors.

The fate of the hybrid cell, i.e., stability of DNA content, tumorigenic and metastatic phenotypes, and resistance to chemotherapy drugs, is now being assessed following in vitro and in vivo passage under both highly selective and relatively unselective conditions.

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REFERENCES


*F. R. Miller, unpublished results.
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