DNA Content of Murine Fibrosarcoma Cell Lines with Varying Metastatic Potential

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ABSTRACT

The DNA content of murine fibrosarcoma cell lines of various metastatic potential was the subject of the current investigation. The cell lines were derived from methylcholanthrene-induced tumors as described previously (J. Varani et al., J. Natl. Cancer Inst., 71: 1281–1287, 1983). Cells were maintained in vitro and used for DNA studies no more than 48 h after passage. DNA staining was accomplished using propidium iodide and flow cytometry was used to quantitate relative amounts of DNA. Trout and chicken erythrocytes and mouse thymocytes were used as internal DNA standards for each cell line. DNA indices were calculated as the ratio of the G0-G1 peak channel number of the tumor cells to the G0-G1 peak channel number of the thymocytes. Manual chromosome counts were also obtained from each cell line using Giemsa-stained preparations. All cell lines demonstrated a single euploid population. The two tumor lines with the highest metastatic potential were slightly hyperdiploid whereas three low metastatic lines were near tetraploid. A sixth line of moderate metastatic potential was also found to be near tetraploid. Chromosome counts and flow cytometric analyses were in close agreement indicating that DNA content was largely due to chromosome replication. These data suggest that, in this model, metastatic potential and DNA content are inversely related once diploidy is exceeded.

INTRODUCTION

The study of metastasis represents one of the most pertinent yet perplexing areas of cancer research. Metastatic dissemination of primary tumors is responsible for the death of the majority of cancer patients, yet metastases may occur early in the growth of the tumor, may be difficult to identify, and may have quite varied characteristics. Early identification of tumors with high metastatic potential may aid in the prevention or cure of the malignant dissemination.

We have developed a murine model for the study of the metastatic process. Using methylcholanthrene-induced fibrosarcomas, cell lines have been selected on the basis of whether or not cell surface α-Galα23 and groups are present, resulting in lines with various metastatic potentials (1, 2). These cell lines have been screened for a variety of characteristics, including metastatic ability, chemotactic and haptotactic responses (3–5), lectin binding (2, 6), cell surface laminin (6, 7), and growth and adherence to a variety of substrates (2, 5, 8). In general, the low metastatic variants are less motile, express less cell surface α-D-galactopyranosyl end groups and laminin, are slower growing, and are less adherent to type IV collagen substrates.

The current study was undertaken to examine the DNA content of these cell lines. Previous studies in both human and animal models have indicated that DNA content may be of potential usefulness in predicting the malignant or metastatic potential of the tumor (9–22). Studies have shown that patients with diploid or near diploid tumors have a much more favorable prognosis than do patients with high ploidy tumors (9–18, 22), although other studies are contradictory to these findings (9, 23, 24). Most of these studies have relied upon resected tumor tissue. Our study relied upon murine cell lines with well documented tumorigenic and metastatic potential in syngeneic animals. Herein we describe the DNA content and chromosome counts of these tumor populations.

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study were derived from three murine fibrosarcomas induced with methylcholanthrene and have been described previously (1, 2). Two high metastatic lines, designated 1.0/L1 and 1.1, a line of moderate metastatic potential, designated 1.2, and three low metastatic lines, 1.0/anti-B', 1.1/anti-B', and 1.2/anti-B', derived from the same three parental tumors were used in this study. All lines were maintained for over 2 years under standard tissue culture conditions in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum. The metastatic potential of each line was assessed periodically by injections of the tumor cells into the footpads of syngeneic mice as described below.

Assessment of Metastatic Potential. To determine the tumorigenicity and metastatic potential of the tumor cell lines, cells were harvested from culture by trypsinization, washed in serum-free RPMI 1640, and injected into the right rear footpads of syngeneic mice. Mice were examined weekly, and 30 days after injection of the tumor cells, tumor sizes were estimated by obtaining measurements of the right rear footpads. The tumor-bearing limbs were then amputated and the mice were sacrificed 14–18 days later. Tissue was removed and inflated with India ink, and metastases were enumerated.

Flow Cytometric DNA Analysis. Cells for DNA analysis were harvested from culture by trypsinization and washed twice in serum-free RPMI 1640 and counted by hemacytometer, and 1 × 10⁶ cells were added per test tube. The following internal standards were added to each test tube: 50 µl chicken RBC (10⁶ cells/ml); 50 µl trout RBC (10⁶ cells/ml); and 40 µl normal mouse thymocytes (1.4 × 10⁶/ml). The cells were pelleted and the supernatant was removed. The cells were then stained for DNA content using a one-step modification of the propidium iodide staining method reported by Vindelov et al. (25). For staining, the cells were resuspended in 1 ml of cold propidium

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2 To whom requests for reprints should be addressed, at Department of Pathology, University of Michigan Medical School, 1315 Catherine Rd., Ann Arbor, MI 48109.
3 The abbreviations used are: α-Galα23, α-Galactopyranosyl; line 1.0/L1, a cloned cell line of high metastatic potential derived from a lung metastasis of a methylcholanthrene-induced fibrosarcoma; line 1.1, an uncultured cell line of high metastatic potential derived from a second methylcholanthrene-induced fibrosarcoma; line 1.2, an uncultured cell line of moderate metastatic potential derived from a third methylcholanthrene-induced fibrosarcoma; lines 1.0/anti-B', 1.1/anti-B', and 1.2/anti-B', low metastatic lines derived from the three methylcholanthrene-induced fibrosarcomas by selection for resistance to the cytotoxic effects of antibodies directed against the human blood group B.
iodide solution [0.01 M Tris, 10 mM NaCl, RNase (0.7 μg/ml) 7 × 10⁻⁵ M propidium iodide, and 0.1% Nonidet P-40, adjusted to pH 8.0], vortexed, and placed in an ice bath for at least 10 min. The stained samples were left in the propidium iodide solution at 4°C until analyzed by flow cytometry. This storage time did not exceed 24 h. Flow cytometric analyses were conducted using a Coulter Epics V flow cytometer (EPICS Division, Coulter Corporation, Hialeah, FL).

Data obtained by flow cytometry was analyzed using the E.A.S.Y. analysis system with the PARA 1 program. This allowed translocation of erythrocyte standards to common channels. The DNA indices of the tumor cell lines were expressed as the ratio of the G₀-G₁ peak channel number of the tumor cell line to the G₀-G₁ peak channel number of the normal mouse thymocytes.

**Chromosome Enumeration.** Chromosomes in each of the fibrosarcoma cell lines were stained using modifications of the method described by Francke and Oliver (26). Briefly 24 h after passage, cells in culture were treated with Colcemid (Grand Island Biological Co.; 0.2 mg/ml, final concentration) for 30 min. Cells were harvested by trypsinization and then subjected to hypotonic treatment and fixation. The fixed cells were applied to microscope slides and dried with heating at 65°C overnight. The slides were then trypsinized, washed, and dried. Finally the chromosomes were stained with MCB Giemsa stain (EM Science, Gibbstown, NJ), washed, and dried. Chromosome counts were then made by counting a minimum of 50 well isolated and defined chromosome spreads per cell line. Results were expressed as the mean ± SD of these counts.

**RESULTS**

**Metastatic Potential of the Cell Lines.** All cell lines were periodically assessed in vivo to determine their metastatic potential. The results of representative experiments are given in Table 1. The metastatic potential and other phenotypic characteristics of the cells were found to be stable over a period of several years (27); however, to ensure uniformity, cell lines were restarted from cryopreserved early passages at approximately 6-month intervals.

**Flow Cytometric DNA Analysis.** Monodisperse nuclei from the tumor cell lines were isolated and stained with propidium iodide as described above. Typical histograms resulting from this type of analysis are presented in Chart 1. DNA indices were calculated in each experiment after translocation of the erythrocyte peaks to a common channel. The means ± SD of the DNA indices for the various tumor lines from replicate experiments are given in Table 2. Our studies revealed that the two tumor cell lines with the highest metastatic potential were slightly hyperdiploid, with DNA indices of 1.15 ± 0.03 (1.0/L1) and 1.10 ± 0.04 (1.1). The low metastatic lines all appeared near tetraploid, with DNA indices of 2.32 ± 0.09 (1.0/anti-B⁺), 2.26 ± 0.02 (1.1/anti-B⁺), and 2.00 ± 0.11 (1.2/anti-B⁺). The last cell line, of moderate malignant and metastatic potential, had a DNA index of 1.89 ± 0.07 (1.2). No significant variations were observed for the DNA indices of the tumor lines over a 1-year period.

**Chromosome Enumeration.** Chromosome enumeration was accomplished by manual counting of well defined chromosome spreads. To be included in the count, a spread had to appear isolated from neighboring cells or spreads and have no overlapping chromosomes. A minimum of 50 chromosome spreads were counted per cell line. Fig. 1 illustrates typical chromosome spreads from high and low metastatic cell lines.

Given the fact that the normal chromosome complement in mice is 40, the DNA indices obtained by flow cytometry were used to determine the number of chromosomes expected in each cell line assuming that chromosome multiplication alone were responsible for the various amounts of DNA. As seen in Table 2, the chromosome counts thus determined were close to those actually observed. In all but one cell line (1.0/anti-B⁺), there...
was an overlap between the means ± SD of the actual and assumed chromosome counts.

**DISCUSSION**

Many previous studies have suggested that low malignant or nonmetastatic tumors are generally near diploid whereas highly aggressive tumors more often appear as aneuploid populations (9-18, 22). In humans, these observations have been reported for patients suffering from tumors of the breast (17), brain (16), prostate (11), lung (18), colon (22), and many other sites (reviewed in Ref. 10). Similarly many investigations using animal models have shown increased ploidy associated with malignancy (19-21). These studies are contradicted by others in which no correlation was observed between ploidy and malignancy (9, 23, 24, 28, 29).

Reports specifically regarding ploidy in metastatic lesions are less frequent than those correlating ploidy with malignancy in general. Those studies which have been reported are contradictory, with some reporting an increased DNA content in metastatic disease (19, 30) and others reporting no significant difference in ploidy between the metastatic and nonmetastatic tumors (20, 23, 25, 29). Investigations into this area seem to be particularly well suited to animal models, since animal tumors can be maintained in vitro, grown or cloned to eliminate normal host cells to precisely karyotype these cell lines in order to determine the intrachromosomal duplication. Currently studies are underway to determine chromosome counts. The tumor cells may contain some chromosomal excesses which cross-react with antibodies specific for the blood group B determinant. Cells which lack the surface α-B-galactopyranosyl groups are resistant to the cytotoxic effect of the antibody. Previous studies with these cell lines (7) suggest that the presence of α-B-Galp end groups on the highly metastatic cells is due in part to a basement membrane glycoprotein, laminin. Laminin is thought to play a role in cell-matrix interactions and may be functionally important in the mechanism of metastasis. Our low metastatic cells, derived from parental tumors by resistance to anti-B antibodies, have previously been shown to be negative for both surface α-B-Galp and laminin (2, 7).

It is unclear whether the high ploidy of the low metastatic cell lines has any causal relationship with the lack of expression of the cell surface components on these cells. It is possible that the great excess of genetic material in the low metastatic line actually reduces a factor or factors responsible for the most malignant phenotype. The current study indicates that the increased DNA is due primarily to chromosome multiplication. It is unclear whether these chromosomal excesses are the result of multiple copies of a single chromosome or replicates of many chromosomes. The slight discrepancies between the predicted and observed chromosome counts may have been the result of technical aberrations in one or both of the methods. On the other hand, it is possible that these discrepancies reflect the fact that chromosome multiplication is not uniquely responsible for the various DNA contents. The tumor cells may contain some chromosomes of abnormal size due to translocations, deletions, or intrachromosomal duplication. Currently studies are underway to precisely karyotype these cell lines in order to determine the exact nature of the chromosomal excesses.

**DNA CONTENT AND METASTATIC POTENTIAL**

Table 2

<table>
<thead>
<tr>
<th>Metastatic potential</th>
<th>DNA index</th>
<th>Chromosome count</th>
<th>Predicted chromosome count</th>
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<tr>
<td></td>
<td>1.0/L1</td>
<td>1.0/anti-B'</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
<td>High/anti-B'</td>
</tr>
<tr>
<td></td>
<td>1.15 ± 0.03</td>
<td>2.32 ± 0.09</td>
<td>1.10 ± 0.04</td>
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<td>High</td>
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**DNA indices (DI) are calculated as**

\[
\text{DI} = \frac{\text{Channel no., } G_2-G_1 \text{ peak of tumor cells}}{\text{Channel no., } G_2-G_1 \text{ peak of normal mouse thymocytes}}
\]

\[\text{a} \quad \text{Expressed as the mean ± SD of three determinations.}

\[\text{b} \quad \text{The normal diploid mouse chromosome complement is 40. Predicted chromosome counts are based upon the assumption that the DNA content obtained by flow cytometry reflects only multiplicity of chromosomes.}

\[\text{c} \quad \text{The normal diploid mouse chromosome complement is 40. Predicted chromosome counts are based upon the assumption that the DNA content obtained by flow cytometry reflects only multiplicity of chromosomes.}

The observation that the cell lines with the highest metastatic potentials were hyperdiploid (an increase of approximately 6 chromosomes) whereas the nonmetastatic clones were hypotetraploid (an increase of approximately 45 chromosomes) is in sharp contrast to the previous studies. The current findings suggest that ploidy and metastatic potential are inversely proportional once diploidy is exceeded. This does not imply that tumor cells of either high or low metastatic potential may not be diploid. Indeed diploid cell clones may have existed in the primary tumor but could have been eliminated by the selection methods used to derive the existing cell lines.

As described previously (2, 31), the low metastatic lines were selected on the basis of resistance to cytotoxicity by antibodies to human blood group B. It is believed that the bases for this selection are the cell surface α-d-galactopyranosyl end groups which cross-react with antibodies specific for the blood group B determinant. Cells which lack the surface α-B-Galp groups are resistant to the cytotoxic effect of the antibody. Previous studies with these cell lines (7) suggest that the presence of α-B-Galp end groups on the highly metastatic cells is due in part to a basement membrane glycoprotein, laminin. Laminin is thought to play a role in cell-matrix interactions and may be functionally important in the mechanism of metastasis. Our low metastatic cells, derived from parental tumors by resistance to anti-B antibodies, have previously been shown to be negative for both surface α-B-Galp and laminin (2, 7).

In the current study well characterized cell lines derived from methylcholanganthrene-induced murine fibrosarcomas were screened for DNA content. The cell lines used were matched pairs of different metastatic potential derived from three distinct primary tumors. All were phenotypically stable with regard to surface markers, growth rates, and metastatic potential.

The investigation using animal models has been less than conclusive in defining a relationship between ploidy and metastasis (19-21). Suzuki et al. (20, 21) published a series of reports using a murine fibrosarcoma model in which higher DNA content appeared to correlate with increased malignancy but not with an increased ability to form spontaneous distal metastases. Using a murine sarcoma system, Reeve and Twentyman (19) observed that spontaneous metastases were most often tetraploid or near-tetraploid, although no attempt was made to compare cloned metastatic isolates to nonmetastatic variants arising from the same parental line. Additional studies involving other rodent tumors found little correlation between ploidy and metastatic potential (28, 29).

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No attempt was made in the current study to utilize flow cytometry to correlate cell cycle status with metastatic potential. In a previous study, manual methods were used to determine that the cell lines with high metastatic potential had greater in vitro growth rates under a variety of conditions than did the lines of lower metastatic potential (2). It is unclear, however, whether growth rates observed in vitro accurately reflect in vivo events. This area is currently under investigation.

An obvious disadvantage of utilizing a single animal model for studies such as these is determining the relevance to observations with other animal models and with clinical material. It is possible that the current observations are pertinent only to this model system and result from the selection procedures used to obtain the cell lines. Interestingly investigations on cell lines derived from primary and metastatic human squamous cell carcinomas (32) have revealed findings similar to those presented here. Thus there is some indication that this murine fibrosarcoma model may be relevant to investigations on human material.

In summary, these data confirm previous observations that tumor populations are frequently aneuploid. Unlike prior investigations, however, the current study suggested that among aneuploid populations of tumor cells a greater DNA content corresponded to a less metastatic phenotype. Whether this relationship is causal or coincidental is unclear and is presently under investigation.

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References

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