Cell Cycle Dependency of Metastatic Lung Colony Formation

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SUMMARY

Exponentially growing FSA 1233 cells, one of the clones isolated from a mouse fibrosarcoma, were synchronized by fractionation according to cell size by centrifugal elutriation. Cells from each fraction were analyzed by flow microfluorometry to determine the stages of the cell cycle and were injected i.v. to determine lung colony-forming efficiency. In vitro plating efficiency of these cells was similar throughout the cell cycle except for a slight reduction at G2 + M. On the other hand, lung colony-forming efficiency showed marked cell cycle and cell size dependencies, being lowest at G1, highest at S, and declining slightly at G2.

INTRODUCTION

Metastasis consists of many steps such as release of cells from a tumor, circulation of tumor cells, lodging at a favorable site, and colonial growth at these new sites. Factors from both tumor cells and host could be involved and interact in all of these steps (2, 3, 5—7, 11, 12, 14, 17—21). In this communication, the latter part of the metastatic process was investigated by lung colony formation after i.v. injection of tumor cells.

The cell FSA 1233 was one of the clones isolated by repeated clonings under selective pressure for high clonogenicity from a methylcholanthrene-induced fibrosarcoma of C3H mouse. This clone grew well both in vivo and in vitro, although the original fibrosarcoma grew very poorly in vitro (10^6 to 10^7 of cloning efficiency). Exponentially growing populations of the cells in vitro were separated into subpopulations in specific phases of the cell cycle by centrifugal elutriation. Using these subpopulations, we examined whether or not metastatic colony formation in lungs depended on stage in cell cycle.

MATERIALS AND METHODS

In Vitro Culture Method. The cells were cultured at 37° in the 5% CO2 atmosphere of an humidified incubator with McCoy's Medium 5A modified by Hsu and containing 20% fetal calf serum. Clonogenicity in vitro (PE)3 was obtained by plating the cells in screw-capped culture tubes with the McCoy's Medium 5A containing soft agar (16). PE's of non-treated control cells were 20 to 50%.

Cell Fractionation and Synchronization by Centrifugal Elutriation. The cells were fractionated by centrifugal elutriation (Beckman JE-6 elutriator rotor) according to sedimentation rate which is related to cell volume. Details of the method have been described (13). In brief, the trypsinized suspension from exponential growth (1.2 x 10⁶) cells from twelve 32-oz bottles, 2 x 10⁶ to 3 x 10⁵/ml) were centrifuged, resuspended in 20 ml elutriation medium, and applied to the machine. The elutriation medium was McCoy's Medium 5A containing 5% fetal calf serum and 5 mM 2-naphthol-6,8-disulfonic acid. The 2-naphthol-6,8-disulfonic acid was added to avoid cell clumping. The entire system was maintained at 4°. Unseparated control cell suspensions were kept in ice during the elutriator run. Fractions Ia and Ib were 35 ml each. The remaining fractions were of 50-ml volumes. The last fraction, i.e., Fraction 14, was a wash fraction. The centrifugation speed was constant at 1210 ± 10 rpm. The medium flow rates were increased by increments of 2 ml/min between Fraction 1b (7.4 ml/min) and Fraction 13 (31.4 ml/min).

Cell Counting and Volume Analysis. Cell count and volume distribution analysis were carried out with a Model ZBI Coulter counter and a Channelizer II multichannel analyzer and plotter (13). The counter was fitted with a 70-μm-diameter, 84-μm-long aperture. The system was calibrated with latex beads (Coulter Electronics, Hialeah, Fla.). The average cell volume for cells in a given sample was calculated from the modal channel number of the volume distribution. For Coulter counting, the cells were suspended and diluted in the medium used for elutriation.

Determination of Cell Stage by Flow Microfluorometric Analysis of Cellular DNA Content. Cell stage was analyzed by monitoring DNA content of cells with FMF (9). Cells were first fixed with 70% ethanol and stained with mithramycin (Mithracin; Charles Pfizer and Co., Inc., New York, N. Y.) for DNA according to the method described by Crissman and Tobey (4), as modified by Barlogie et al. (1). The staining solution contained mithramycin, 50 μg/ml, and 7.5 mM MgCl₂ in 12.5% aqueous ethanol. The flow microflurometer was of the same type as that described by Steinkamp et al. (15). A laser wavelength setting of 457 nm was used to excite the dye. All FMF measurements were performed in the Physics Department at the University of Houston. The fractions of cells in the G1, S, and G2 + M phases of the cell cycle were determined by a computer fit analysis of the DNA

[^1]: This study was supported in part by the Department of Health, Education, and Welfare, NIH, National Cancer Institute Grants CA-11138, CA-06294, CA-18628, and CA-17364. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and in accordance with current United States Department of Agriculture and Department of Health, Education, and Welfare, NIH, regulations and standards.

[^3]: The abbreviations are: PE, plating efficiency; FMF, flow microfluorometry; LCFE, lung colony-forming efficiency.
distribution using a mathematical method of Fried (8).

**Mice and Lung Colony Formation.** Male C3Hf/Bu mice from our specific-pathogen-free breeding colony were 8 to 10 weeks old at the beginning of each experiment. Animals were maintained on a sterilized pellet diet and sterile water.

The assay method for LCFE has been described elsewhere (6, 9-12, 17, 18, 20). In this work, single-cell suspensions of 10^5 cells/mouse in 0.5 ml of medium were injected without addition of microspheres or heavily irradiated cells into tail vein of unirradiated and untreated mice (10 mice/group). The mice were sacrificed 19 days later. The lungs were removed and fixed in Bouin's solution. Colonies were scored by the naked eye.

**RESULTS**

After fractionation by centrifugal elutiation (Chart 1), cells were centrifuged and resuspended in fresh medium at a concentration of 2 \times 10^5/ml. Cells from an unseparated control and Fractions 3, 5, 7, and 9 (Experiment 4) or Fractions 4, 6, 8, and 10 (Experiments 5 and 6) were examined for the ability to form lung colonies in C3H mice, for the ability to form colonies *in vitro*, and for their relative position in the cell cycle. The DNA distribution patterns of cells in the fractions (Experiment 6) obtained by FMF analysis are shown in Chart 2. An experimentally obtained DNA histogram as well as the computer-fitted curve are presented for comparison in Chart 3. The LCFE *in vivo*, the PE *in vitro*, and the computed results of the cell cycle analysis from 3 separate experiments are summarized in Table 1. Chart 4 summarizes the relationship between LCFE and size or cycle parameters of cells collected in each of the fractions. Routinely, cells collected in Fractions 3, 4, and 5 were primarily in G1; those in Fraction 6 were in G1 + S; cells in Fraction 7 were in S; those in Fraction 8 were in S + G2; and cells in Fractions 9 and 10 were in G2 + M phases of the cell cycle. The average cell size increased 2-fold (linear in semilog plot) for cells collected at Fraction 10, as compared to Fraction 3. The PE's of these cells were relatively constant throughout the cell cycle except for a slight reduction in Fraction 10. However, LCFE varied significantly among cells collected in various fractions. This difference ranged from 6- to 20-fold depending on the fractions compared. In Experiments 5 and 6, Fractions 4 (75% G1, with 23% contamination of S) and 6 (47% S, mostly early S with 44% G1, contamination) were compared, and they differed 6-fold; while in Experiment 4, Fractions 3 (90% G1, with 9% contamination of S) and 7 (47% S, mostly mid-S with 24% G1, contamination) were compared, and they differed 20-fold. Fractions 3 and 7 were much more representative for G1 and S than were Fractions 4 and 6. S-phase cells appeared to be much more efficient in forming lung colonies than did G1 cells. In these 3 experiments, plus 3 additional experiments, cells in Fractions 8 or 9 had a lower LCFE than those in Fractions 6 or 7. This result indicates that cells in mid-S appear to have a greater LCFE than do late S-G2-phase cells.

**DISCUSSION**

In this communication we have demonstrated the usefulness of the methods of centrifugal elutriation and FMF in determining the relationship(s) between the parameters of size and position in the cell cycle and the clonogenic and/or metastatic ability of fibrosarcoma cells.

Large cells (S, G2, M) appear to be more efficient in forming lung colonies than do small cells (G1). LCFE increased markedly with progression of the cells from G1 to S, with S-phase cells being the most efficient. These phenomena, however, are not due to differences in clonogenic ability, since cells in all fractions exhibited similar PE's *in vitro*. 

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**Chart 1.** Fractionation of FSA 1233 cells by centrifugal elutriation. The modal volume may be calculated by multiplying the modal channel number by 56 cu μm.

**Chart 2.** DNA distribution pattern of fractions (Fract.) analyzed by FMF. Abscissa, channel number (fluorescence intensity, DNA content).
Table 1

<table>
<thead>
<tr>
<th>Lung colonies/10⁶ cells</th>
<th>In vitro PE (%)</th>
<th>LCFE/viable cell*</th>
<th>Composition of fractions with respect to cell cycle phase (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G₁</td>
</tr>
<tr>
<td>Unseparated control</td>
<td>1.0 ± 0.4</td>
<td>18.3 ± 1.1</td>
<td>5.5 × 10⁻⁴</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>0.9 ± 0.4</td>
<td>18.7 ± 1.5</td>
<td>4.9 × 10⁻⁴</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>2.8 ± 1.4</td>
<td>17.5 ± 1.1</td>
<td>1.6 × 10⁻⁴</td>
</tr>
<tr>
<td>Fraction 7</td>
<td>17.6 ± 4.2</td>
<td>18.4 ± 1.1</td>
<td>9.6 × 10⁻⁴</td>
</tr>
<tr>
<td>Fraction 9</td>
<td>6.3 ± 2.4</td>
<td>16.7 ± 1.1</td>
<td>3.8 × 10⁻⁴</td>
</tr>
</tbody>
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Experiment 5

| Unseparated control    | 1.8 ± 1.0         | 2.2 ± 0.4         | 8.2 × 10⁻⁴ | 48   | 28   | 24    |
| Fraction 4             | 0.8 ± 0.3         | 3.6 ± 0.4         | 6.3 ± 10⁻⁴ | 79   | 49   | 2     |
| Fraction 6             | 4.6 ± 1.7         | 1.8 ± 0.4         | 6.3 ± 10⁻⁴ | 43   | 45   | 12    |
| Fraction 8             | 2.6 ± 1.2         | 2.7 ± 0.4         | 6.9 ± 10⁻⁴ | 16   | 41   | 43    |
| Fraction 9             | 3.9 ± 2.2         | 2.2 ± 0.4         | 1.8 × 10⁻³ | 7    | 31   | 62    |

Experiment 6

| Unseparated control    | 11.2 ± 4.0        | 12.1 ± 0.8        | 9.3 × 10⁻⁴ | 43   | 29   | 28    |
| Fraction 4             | 4.4 ± 1.7         | 8.3 ± 0.6         | 5.3 ± 10⁻⁴ | 71   | 27   | 2     |
| Fraction 6             | 27.9 ± 7.6        | 11.1 ± 1.0        | 2.5 × 10⁻⁴ | 45   | 49   | 6     |
| Fraction 8             | 22.6 ± 11.8       | 9.7 ± 1.1         | 2.3 × 10⁻⁴ | 10   | 39   | 51    |
| Fraction 10            | 26.0 ± 15.8       | 7.7 ± 0.7         | 3.4 × 10⁻³ | 7    | 17   | 76    |

* Calculated by dividing the LCFE by PE/100.
* Mean ± S.E.
* Mean ± S.E. among 8 tubes.

Chart 3. Computer fit analysis used in the calculation of the percentage of cell cycle stage of a sample. Abscissa, channel number (DNA content). - - - -, actual data (unseparated control of Experiment 6) by FMF. -----, curves fitted by computer. A, synthesized curve compiled from component curves (B) that were used for computation.

Cell volume increased linearly in a semilog plot by a factor of 2 between the G₁ and G₂ + M fractions (Fractions 3 and 10), while LCFE differed on the average of 10-fold between fractions representing G₁ and S (Chart 4). This indicates that position in the cell cycle might have an important role rather than a simple mechanical size effect.
Cell Cycle Dependency of Metastasis

Fraction Number

Chart 4. Relationship between LCFE and cell size or cell cycle stage. LCFE (top), cell volume (middle), and percentage of cells in G₁, S, and G₂ + M stage (bottom) are summarized along with fraction number (abscissa). LCFE is expressed as (LCFE/survivor of each fraction)/(LCFE/survivor of the unseparated control). The cell volumes were calculated by multiplying the channel number by 56 cu µm. Both LCFE and cell volume are plotted on semilogarithmic scale against fraction number. Percentages of cells in each stage of cell cycle are plotted in linear scale.

It is apparent that the ability of cells to form lung colonies is not a simple reflection of the clonogenic ability of the cells but is dependent on additional parameters. These parameters might include cell size or other intrinsic properties such as cell surface and/or intracellular properties (2, 3, 6, 19). If cell surface factors are considered, it must be noted that the trypsinization used for preparation of the cell suspensions alters the surface structure.

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REFERENCES

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