Effects of Folate Deficiency on the Metastatic Potential of Murine Melanoma Cells

Richard F. Branda, John J. McCormack, Carol A. Perlmutter, Linda A. Mathews, and Steven H. Robison

ABSTRACT

Experiments were designed to measure the effect of folic acid deficiency on the growth of melanoma cells in vitro. Malignant melanoma cells (F10 strain) were grown in folate-deficient and -supplemented media. After 3 days, cells in the deficient medium had restricted proliferative capacity, low folate levels by bioassay, increased cell volume, abnormal deoxuryridine suppression tests, accumulation of cells in S phase by flow cytometry, and increased numbers of DNA strand breaks. These folate-deficient cells consistently initiated more pulmonary metastases than control cells when injected into host mice. Cell size did not appear to be a major factor in pulmonary metastasis formation. In vitro growth rates and cloning efficiencies were comparable for cells in both types of medium as was subcutaneous growth of tumors. We conclude that folate deficiency increases the metastatic potential of cultured melanoma cells.

INTRODUCTION

Malnutrition is frequently observed and is often severe in patients with malignant diseases (1). It is due to the combined effects of decreased nutrient intake and increased utilization by tumor metabolism. Losses of adipose tissue and muscle protein are common, as is weight loss; the latter may adversely affect survival and response to chemotherapy (1). Deficiencies of micronutrients are also frequently encountered in cancer patients. For example, some nutritional surveys have reported low blood levels of folic acid in 60 to 85% of cases (2, 3). In the series reported by Magnus, 11 of 64 patients with metastatic cancer had serum levels of 0.9 ng/ml; 19 others had levels of 1 to 1.9 ng/ml; 53 of these 64 patients had serum levels below the normal range (3.0 to 11.0 ng/ml) for his laboratory (2).

Despite its frequency in patients with malignancies, the effect of folic acid deficiency on the clinical course of neoplasia has not been studied in detail. Early reports by Farber and coworkers of an "acceleration phenomenon" in leukemic patients receiving folic acid were followed by animal studies suggesting that tumor growth is retarded in folate-deficient hosts (4, 5). Subsequently clinicians have been reluctant to administer folic acid to deficient cancer patients on the chance that the vitamin might enhance the growth of the malignancy. Since the publication of these studies 20 or more years ago, a great deal has been learned about folate metabolism. Consequently, we sought to investigate the influence of this common vitamin deficiency on tumor biology in the light of more recent information. Folic acid metabolism critically affects chromosome stability, and vitamin lack is associated with a variety of chromosomal abnormalities (6-9). Since tumor metastatic propensity appears to correlate with chromosomal instability (10), we investigated the effect of cellular folate deficiency on this important determinant of tumor lethality.

MATERIALS AND METHODS

Murine B16 melanoma cells (F10 strain) were obtained from the Tumor Repository of the Division of Cancer Treatment, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. The cells were grown in "folate-free" Dulbecco's modified Eagle's medium (M. A. Bioproducts, Walkersville, MD) or the same medium supplemented with 4 µg/ml of folic acid (Sigma). To these media were added 10% dialyzed horse serum, 2% glutamine, and 1% penicillin-streptomycin (all obtained from Gibco Laboratories, Grand Island, NY). The final "low-folate" medium contained 0.26 ng/ml of folate activity by the Lactobacillus casei assay (11, 12). Cells suspensions in 15 ml of medium were placed in T75 flasks (Corning) and incubated at 37°C with 5% CO2. Cell counts were performed manually and viability was assessed by trypan blue exclusion. Cell cultures were divided by treating for 5 min with 2 ml of Versene (1:5000), washing with medium, and inoculating a new flask with 1 x 10⁶ cells. In some experiments, flasks were inoculated with 5 x 10⁵ cells.

Intracellular folic acid levels were measured with a L. casei assay (13). Melanoma cells were treated with Versene and washed with cold saline. Then 1 x 10⁵ cells were suspended in 2 ml of 0.1 % sodium acetate buffer (pH 4.5) containing 0.1% ascorbate and 0.2 ml of dialyzed horse serum. This suspending medium contains no measurable folate activity. Following sonication and incubation of the homogenate for 90 min at 37°C, the protein was denatured by boiling for 5 min and removed by centrifugation. The supernatant was assayed as previously described (11, 12).

Deoxuryridine suppression tests were performed by modifications of our previously reported methods (12). After Versene treatment and washing with medium, melanoma cells were resuspended in appropriate medium at a concentration of 1 x 10⁶ cells/ml. One ml of suspension was placed in 12- x 75-mm tubes, to which was added deoxuryridine (Sigma) (final concentration, 0.1 or 1 µM) in phosphate-buffered saline or buffer alone. After 1 h at 37°C, 0.1 µCi of [³H]thymidine (Research Products International Corp., Mt. Prospect, IL) was added, and the mixture was incubated for an additional 3 h. Radioactivity was then determined in the trichloroacetic acid precipitates. Results are expressed as thymidine incorporation in samples with deoxuryridine divided by incorporation by control cells in deoxuryridine-free cultures x 100. All determinations were done in triplicate.

Cell cycle distribution was determined by flow cytometry and analyzed by a parametric method (14). Melanoma cells (1 x 10⁶) were stained with propidium iodide (50 µg/ml in 10% horse serum containing 1 mg/ml of RNase and 0.025% Nonidet P-40). Nuclear fluorescence intensity was determined with an Ortho Cytoflouorograf 50 H/H using 488-nm argon ion laser excitation.

DNA strand breaks were detected by alkaline elution as described by Kohn with several minor modifications (15, 16). Cells were seeded at 1 x 10⁶/ml in 10 ml of medium containing 0.02 µCi/ml of [¹⁴C]thymidine for 24 h. The cells were deposited on a polycarbonate filter (2-µm pore size) and lysed with a buffer containing 2% sodium dodecyl sulfate-20 mM EDTA (pH 10.1). The filters were washed with 5 ml of 20 mM EDTA (pH 10.1). Elution buffer (25 ml of 20 mM EDTA (free acid)-
2% tetrapropyl ammonium hydroxide (pH 12.15) was applied to the filter and pumped through at a rate of 0.035 ml/min. Fractions of 3 ml were collected at 90-min intervals. Afterward, the filter was dissolved in 1 ml of protosol and prepared for liquid scintillation counting. Elution fractions and a wash of the pump lines were suspended as a gel using Aquasol-2 containing 0.7% glacial acetic acid and assayed for radioactivity.

Cloning efficiency was measured by growing cells for 3 days in standard or low folate medium and then plating at a density of 100 or 200 cells/plate in 60-mm culture dishes containing standard culture medium. After 1 wk, the plates were stained with crystal violet and colonies were counted.

Mean channel volume was measured on a Model ZBI Coulter Counter and Model 256 channelyzer (Coulter Electronics, Inc., Hialeah, FL). The mean channel volume was converted into mean volume by calibrating the machine with microspheres of 9.77, 14.75, and 20.01 μm in diameter (Epics Division of Coulter Corp., Hialeah, FL).

Chromosome counts were performed on cells which were incubated with Colcemid (0.02 μg/ml) for 2 h prior to harvesting. Following Versene treatment, the suspended cells were treated with 0.075 M KCl for 15 min at 37°C, fixed with methanol:acetic acid, 3:1 by volume, for 10 min, spread on wet slides, and then air dried. Slides were stained with 2% Giemsa solution.

Metastasis formation was determined by injecting murine melanoma cells into the tail veins of male C57BL/6 mice which were 6 to 8 wk old. The cell suspension contained 1 × 10⁶ viable cells in 0.1 ml of medium. Cell viabilities were 95.7 ± 3.4% (mean ± SD) for control cells and 95.4 ± 3.0% for low folate cells in 13 experiments.

RESULTS

Murine B16 melanoma cells were grown in folate-deficient Eagle's medium or the same medium supplemented with folic acid. In both types of medium, there is a 1-day lag in growth, while the cells adhere to the flask, followed by a 2- or 3-day period of exponential growth (Fig. 1). If the cells remain confluent they quickly lose viability over the next 1 or 2 days. If, however, the cells in standard medium are divided and cultured in fresh medium on Day 3, they will again grow exponentially after a 1-day lag. Cells grown in folate-deficient medium proliferate at the same rate as control cells until confluence (Fig. 1, Days 1 to 3). If these cells from folate-deficient medium are divided into folate-supplemented medium after 3 days of culture, they will proliferate at the same rate, or slightly faster, than control cells (Fig. 1, Δ). On the other hand, if they are split into fresh, low folate medium, they do not proliferate (Fig. 1, O; Days 3 to 6). These results suggest that folate deficiency reversibly limits cell growth after approximately 3 days in low folate medium. The reversible nature of this inhibition is further substantiated by the observation, that in 10 experiments, the cloning efficiencies of cells after 3 days in control or low folate medium were similar, 70.5 ± 7.9 and 58.4 ± 6.5 (mean ± SE), respectively. This difference was not statistically significant.

As shown in Fig. 2, mean cell size increased progressively when melanoma cells were grown in culture for 3 to 5 days, but this increase was greatly magnified by growing the cells in folate-deficient medium. The latter cells also manifested a greater variation in cell size, indicated by the width of the curves. After 3 days in culture, the differences were rather modest. In 6 experiments the mean cell volume ± SD for control cells was 1627 ± 96 fl, while for folate deficient cells the mean was 2023 ± 206 fl. After 4 days of incubation, the differences were more substantial: 1825 ± 165 fl (control); 2539 ± 439 (low folate). Cells which survived for 5 days in folate-deficient medium were very large: 1912 ± 238 fl (control) compared to 2759 ± 799 fl (low folate).

To assess the folate status of these cells more directly, intracellular folate activity was determined with a L. casei assay, and deoxyuridine suppression tests were performed (Table 1). The latter test measures the availability of folate compounds for conversion of deoxyuridine to thymidine. Homogenates of cells grown in standard medium contained L. casei activity which fluctuated between 0.38 and 0.95 ng/10⁶ cells. These values represent the mean of 6 determinations. The levels of folate activity appeared to vary with growth rate, being lowest during the exponential phase. In contrast, melanoma cells grown in low-folate medium had a drop in folate activity during the culture period to a nadir of 0.28 ng/10⁶ cells. Deoxyuridine suppression tests were performed as paired observations in at least 6 experiments, using two different concentrations of deoxyuridine (0.1 and 1 μM). After 48 h of culture, deoxyuridine suppression in cells from low-folate medium tended to be reduced compared to control cells, but these differences were not statistically significant. However, after 72 h in folate-
deficient medium, melanoma cells manifested abnormal deox-
yuridine suppression tests at both concentrations of deoxy-
yuridine (P < 0.05 by the paired t test) (17). Thus, by 72 h of culture in deficient medium, melanoma cells had sufficiently low intracellular levels of folate activity to limit formation of thymidylate for DNA synthesis (Table 1).

As an additional index of folate activity and its effect on DNA metabolism, cell cycle distribution in 5 separate cultures was determined by flow cytometry (Table 2). Distribution was similar until 72 h of culture, when highly significant differences were noted in the numbers of cells in G0-G1 and S in low folate medium. Therefore, by 3 days of incubation, cells in low folate medium have an accumulation of DNA synthesis in S phase compared to control cells.

The effect of this folate deficiency and impaired DNA synthesis on melanoma chromosomal stability was assessed by the alkaline elution test. In this assay, decreased retention of DNA on the filter indicates the presence of DNA strand breaks. After 2 days of incubation, cells in low-folate and standard media had similar elution rates (P > 0.05). However, by 3 days there was a statistically significant decrease (P < 0.01) in retention of DNA from folate-depleted cells, and this effect became much more pronounced by 4 days in low folate medium (Fig. 3).

Karyotypic analysis indicated that the F10 cell line was highly aneuploid. The mean chromosome number in 30 metaphases was 68.1, with a range of 42 to 78. Results in folate-deficient cells were nearly identical: mean, 68.0; range, 48 to 77.

Taken together, these studies indicate that, after 3 days of culture in folate-deficient medium, murine B16 melanoma cells have a reduced proliferative capacity, increased cell volume, low intracellular folate levels, impaired utilization of folate compounds for thymidine synthesis, accumulation of cells in S phase, and increased fragility of DNA compared to cells in folate-supplemented medium.

Murine melanoma cells were again grown for 3 days in standard and low folate media and injected as a suspension (1 × 10⁶ viable cells in 0.1 ml of medium) into the tail veins of normal C57BL/6 mice. Control (standard medium) cells were always injected first (18). Pigmented pulmonary and hepatic metastases were scored 10 to 14 days later. Folate-deficient cells initiated more pulmonary metastases compared to control cells. Following a 3-day incubation in folate-deficient medium did not permanently alter proliferative capacity. Tumor cells from this experiment in folate-deficient medium formed 126 (85 to 180), 96 (47 to 300), and 39 (20 to 60) pulmonary metastases, since cells cultured for 2 days did not show enhanced metastasis formation (92 versus 66, and 5.8 versus 5.6, control versus cells in low folate medium; these differences were not statistically significant). We conclude that attention to culture conditions can reduce but not eliminate the variability in assay results.

Cell size alone did not appear to have an important effect on metastasis formation. As shown in Fig. 2, mean cell size increased progressively during incubation in folate-deficient medium. Nevertheless, there was not a coincident change in metastatic capacity of tumor cells. Finally, it was important to incubate the tumor cells for at least 3 days in folate-deficient medium, since cells cultured for 2 days did not show enhanced metastasis formation (92 versus 66, and 5.8 versus 5.6, control versus cells in low folate medium; these differences were not statistically significant). We conclude that attention to culture conditions can reduce but not eliminate the variability in assay results.
Fig. 3. DNA strand breaks detected by alkaline filter elution in melanoma cells (F10 strain). Cells were cultured in standard (◊) or folate-deficient (O) medium for the indicated number of days. Then cells were deposited on a filter and lysed, and the DNA eluted as described in the text. At the indicated number of hours of elution, retention of radiolabeled DNA was determined. Points, mean of quadruplicate determinations; bars, SEM.

Table 3  Metastatic potential of B16 murine melanoma cells after incubation in standard or low folate medium

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Median no. of nodules/lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard medium</td>
</tr>
<tr>
<td>1</td>
<td>14 (10-198)</td>
</tr>
<tr>
<td>2</td>
<td>23 (9-78)</td>
</tr>
<tr>
<td>3</td>
<td>210 (68-318)</td>
</tr>
<tr>
<td>4</td>
<td>11 (9-19)</td>
</tr>
<tr>
<td>5</td>
<td>14 (0-54)</td>
</tr>
<tr>
<td>6</td>
<td>17 (2-53)</td>
</tr>
<tr>
<td>7</td>
<td>25 (5-134)</td>
</tr>
<tr>
<td>8</td>
<td>56 (3-401)</td>
</tr>
<tr>
<td>9</td>
<td>10 (0-43)</td>
</tr>
<tr>
<td>10</td>
<td>38 (23-75)</td>
</tr>
<tr>
<td>11</td>
<td>47 (28-108)</td>
</tr>
<tr>
<td>12</td>
<td>15 (2-28)</td>
</tr>
<tr>
<td>13</td>
<td>51 (15-305)</td>
</tr>
</tbody>
</table>

* n, number of animals per group.
** Significance of difference between groups by the Mann-Whitney U test.

Fig. 4. Subcutaneous growth of B16 melanoma (F10 strain) cells following a 3-day incubation in either standard or folate-deficient medium. Points, mean of 9 animals given injections of cells from either low folate (C) or control (◊) medium. Bars, SEM. The differences between points on the two curves were not statistically significant.

Fig. 5. Cell cycle distribution in cells cultured in folate-replete medium following incubation in either standard or folate-deficient medium. Melanoma cells (F10 strain) were cultured for 3 (left and middle) or 5 days (right) in either standard medium (left) or folate-deficient medium (middle and right). All cells were then transferred to standard (folate-replete) medium. Cell cycle distribution was determined 24 and 48 h later.
anoma cells have an enhanced capacity to colonize and form metastases. The mechanism by which folate deficiency increases metastatic capacity or, more specifically, organ colonization is unclear at present.

Our studies confirm the work of others showing that folate deficiency restricts the proliferation of rapidly dividing cells (20). Initially, this restriction is reversible, but after 5 days in low folate medium, the melanoma cells are unable to regain proliferative capacity with folate supplementation. Thus, severe folate deficiency resembles treatment with antifolts, such as methotrexate, and eventually leads to cell death. On the other hand, there was only a slight tendency, which was not statistically significant, for folate replacement to accelerate the growth of deficient tumor cells (Fig. 4). Therefore, it appears that replacing folate levels of deficient cells restores proliferative capacity but does not increase the rate of proliferation above the usual doubling time of the tumor (13). Based upon this observation, it seems unlikely that the increased metastatic potential of folate-deficient cells is due simply to accelerated growth in a folate-replete host and earlier recognition of lung nodules.

Metastasis formation is a multistep process, and folate deficiency could conceivably alter one or more of these steps. Folic acid is necessary for 1-carbon transfers and participates in amino acid, purine, and pyrimidine synthesis. Deficiency of the vitamin produces large cells with delayed nuclear maturation and an imbalance between DNA and RNA synthesis. These megaloblastic cells might be more likely to arrest in the pulmonary capillary bed, but this mechanical alteration would not explain the increased colonization of other organs by folate-deficient cells. We did not find that metastatic capacity increased substantially when cells were grown for as long as 5 days in folate-deficient medium, despite the fact that these cells were much larger, on average, and with a greater variability in size, than folate-replete cells. Moreover, the correlation between mean cell volume and metastasis formation was weak when analyzed in 7 experiments. These observations are consistent with studies in other laboratories of hydroxyurea-treated tumor cells. Pretreatment with hydroxyurea of 3 murine tumor cell lines (B16-F1, B16-F10, and K-1735-clone 19) brought about a marked increase in the median number of lung tumor nodules. Treatment with 0.1 mM hydroxyurea was associated with little change in cell volume but significantly increased metastatic propensity. In other experiments, hydroxyurea-treated cells were larger than controls, but they were not trapped in the lungs to a greater extent than control cells. These authors concluded that neither cell size nor the position of the majority of the cells in the cell cycle was a major factor in pulmonary metastasis formation (21).

It seems more likely that the increased metastatic potential of folate-deficient cells is related to the effects of folate compounds on nucleic acid metabolism and chromosome stability. Decreased intracellular folate activity results in profound alterations in nucleotide pools (22, 23). Under these conditions, there is a reduction in the rate of DNA replication fork movement and inhibition of the gap-filling step and/or joining of Okazaki pieces, resulting in DNA with persistent single-stranded regions (24). In addition, dUMP tends to be incorporated during gap repair, leading to its cyclic incorporation and removal.

This process may result in extensive degradation of DNA (23). Since one of the DNA polymerases responsible for the repair of DNA damage is thought to be involved with DNA replication, it is likely that the repair process is also affected. Finally, DNA ligase, an enzyme common to the repair and replication processes, is inhibited by a rise in the intracellular pool of dATP as occurs in folate deficiency (22). These metabolic alterations could account for the chromosome abnormalities observed in patients with megaloblastic anemias. Chromosome breaks, gaps, desprialization, and increased sister chromatid exchanges have been reported (6–8). That normal folic acid metabolism is necessary for the integrity of chromosomes is further suggested by reports that folate-deficient medium enhances expression of fragile sites and that methotrexate causes chromosomal abnormalities (9, 25). Taken together, these observations indicate that folate deficiency is associated with chromosomal instability.

Increased genetic instability contributes to the generation of tumor cells with the capacity to metastasize, presumably by fostering diversity. Only a few cells in a primary tumor have the combination of characteristics required to progress through the complex series of steps necessary to form a metastasis (26). These variants arise due to an acquired genetic variability or instability in tumor cells which allows diversity to develop with time (27). In vivo host selection pressures then determine which variants survive and grow. Recent studies suggest that cell lines with greater metastatic ability have a higher rate of generation of metastatic variants (10). Metastatic potential can be further enhanced by exposure to mutagens such as ultraviolet radiation (28). Pretreatment of murine melanoma cells with chemotherapeutic agents has been associated with increased metastatic capacity. Hydroxyurea, 1-β-D-arabinofuranosylcytosine, 5-aza-cytidine, and methotrexate all produced significant increases in the number of lung metastases (21, 29). The latter drug is particularly relevant to our studies, because of the well-known effects of methotrexate on folate metabolism. We propose that folate deficiency, by increasing chromosomal instability, might similarly promote tumor heterogeneity and increase the likelihood of developing cells with the capacity to form metastases. We did not find that major karyotypic abnormalities were induced by folate deficiency in this highly aneuploid cell line. This observation is consistent with studies in other laboratories which found no apparent relationship between generation of metacentric marker chromosomal changes and metastatic potential. These authors proposed that more subtle nonrandom genetic or molecular changes may be determining factors for malignant potential (30). Our observation that folate-deficient melanoma cells have more DNA strand breaks than folate-replete cells (Fig. 3) may implicate these chromosomal changes in the process which eventuates in metastatic variants. These associations do not prove a causal relationship, but they provide a hypothesis that can be tested further.

REFERENCES

FOLATE DEFICIENCY AND METASTATIC POTENTIAL

Effects of Folate Deficiency on the Metastatic Potential of Murine Melanoma Cells


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/16/4529

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.