Effect of Folate Deficiency on Mutations at the hprr Locus in Chinese Hamster Ovary Cells Exposed to Monofunctional Alkylating Agents

Richard F. Branda, Amy R. Lafayette, J. Patrick O’Neill, and Janice A. Nicklas

Abstract

Multiplex PCR amplification of hprr exons from 113 Chinese hamster ovary cell clones selected for resistance to 6-thioguanine was performed to investigate the molecular basis for the synergistic mutagenic effects of nutritional folic acid deficiency and alkylating agents. In cells treated with ethyl methanesulfonate, intragenic deletions were detected in 9 of 46 (19.6%) clones derived from folate-deficient cells, but in none of 16 mutants grown in folate-replete medium. The number of deletions found in mutants generated by N-nitroso-N-ethylurea was low in both folate-deficient (1 of 25; 4%) and folate-replete (1 of 26; 3.8%) cells. Correction of folate deficiency may decrease the frequency of intragenic deletions caused by some alkylating agents.

Introduction

Nutritional deficiency of folic acid in vivo is associated with numerous types of genetic damage. Cytogenetic abnormalities that have been reported include chromosomal gaps, breaks, triradial, quadriradial, and ring forms, allocyclic chromosomes, expression of fragile sites, increased rates of sister chromatid exchanges, and elevated frequencies of erythrocyte micronuclei (1, 2). On a molecular level in vivo, DNA strand breaks (1, 3) and hypomethylation within the p53 tumor suppressor gene (3) have been described in folate-deficient cells. We (4) previously found that the combination of folate deficiency and monofunctional alkylating agents (EMS or ENU) or γ-irradiation was synergistic in producing DNA strand breaks as well as in augmenting somatic mutations in CHO cells in vitro, as detected by measuring the frequency of TG-resistant cells or by the diphtheria toxin resistance assay. The mechanism(s) by which folate deficiency enhances somatic mutations after exposure to mutagens/carcinogens are unclear at present. Possibilities (which are not mutually exclusive) include disruption of DNA repair, enhanced sensitivity to endonucleases due to hypomethylation, cytosome-to-thymine transition mutations at the site of demethylated cysteine residues, repetitive insertion and excision of uracil bases in place of thymidine leading to strand breaks, decreased “gap-filling” DNA synthesis, and deoxynucleotide pool imbalances (1, 5, 6). The molecular analysis reported here was performed to gain insight into the synergy between folate deficiency and the mutagenic effects of monofunctional alkylating agents.

Materials and Methods

Cell Culture. After incubation with ENU or EMS, replicate cultures of cells were established in 25-cm² flasks. After 6–8 days of growth for phenotypic expression, mutants were selected by plating at 2 × 10⁵ cells per 100-mm dish in 10 µM TG. Only one colony was then isolated from each replicate culture to ensure that independent mutations were analyzed. These mutants came from five independent treatments, in which 5–10 replicate cultures were established from each treated cell population. Mutant clones were picked and stored in liquid nitrogen for future analysis, at which time they were quick-thawed in a 37°C water bath and added to Ham’s F-12 medium (JRH Biosciences, Lenexa, KS) in a 75-cm² tissue culture flask. Flasks were placed in a 37°C, 5% CO₂ incubator, and the cells were allowed to attach overnight. The next morning, fresh medium was added, and the cells grew for 3–4 days until they reached confluency. Each sample was then trypsinized, pelleted, and counted, and several aliquots were prepared of different cell densities, flash-frozen in liquid nitrogen, and stored at −70°C (six 0.5-ml tubes at 10⁶ cells/tube and a 15-ml tube of 1–5 million cells for DNA isolation and purification). The remaining cells were cryopreserved and stored under liquid nitrogen.

Multiplex PCR. All nine exons of the hprr gene were simultaneously amplified in a multiplex PCR amplification (7). Genomic DNA was isolated and purified using a standard phenol-chloroform method, and ~200 ng of DNA were added to a cocktail of 16 primers (Middle Certified Reagent Co., Miami, TX, Table 1) 25 µm each dNTP, 10% DMSO, 6.7 mM MgCl₂, high-performance liquid chromatography-treated water, and Gitscher buffer [670 mM Tris-HCl (pH 8.8), 166 mM (NH₄)₂SO₄, 50 mM 2-mercaptoethanol, and 68 µM EDTA]. A 95°C hot start denaturation step was performed initially for 5 min, and the samples were placed on ice before 3 units of Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT) were added. A multiplex profile was modified for use on the Perkin-Elmer 2400 thermal cycler but was also successfully run on the Perkin-Elmer 480 and Ampliton II thermal cyclers. The samples were subjected to 30 cycles of 68°C for 20 s (denaturation), 94°C for 20 s (annealing), and 55°C for 30 s (extension) and a final extension of 68°C for 7 min. PCR product (20 µl) with 5 µl of loading dye (bromphenol blue and xylene cyanol) were loaded into a 3% NuSieve gel (FMC Bioproducts, Rockland, ME) in 1× Tris-borate EDTA buffer and run at 100 V for ~2.5 h and stained with ethidium bromide.

Results and Discussion

The CHO cell clones analyzed in this report were derived from previously reported experiments (4). Table 2 shows the mutant frequencies for CHO cells cultured in folate-replete or -deficient medium and treated with EMS or ENU, compared to those of cells treated with diluent alone. EMS increased mutant frequencies about 28-fold in folate-replete cells and about 47-fold in folate-deficient cells. ENU-treated cells had 36- and 81-fold increases in folate-replete and -deficient media, respectively. Therefore, it is highly unlikely that any of the analyses were performed on background, as opposed to induced, mutants. As shown in Table 3, hprr gene multiplex amplification of TG-resistant CHO genomic DNA identified nine deletions in 46 mutants grown in folate-deficient medium and treated with EMS, but no deletions among 16 mutants grown in folate-replete medium and treated with EMS. In contrast, the numbers of deletions found in mutants generated by ENU were similarly low in folate-deficient (1 of 25) and folate-replete (1 of 26) CHO cells. These results suggest that the synergistic augmentation of EMS-induced mutations at the hprr locus by folate deficiency is frequently due to deletion events, whereas the interaction of ENU and folate deficiency appears to result primarily in increased numbers of base changes.
induced mutant clones derived from folate-deficient CHO cells is reminiscent of studies performed by Op het Veld et al. (13) with the Chinese hamster cell line EM-C11. This line is highly sensitive to the cell killing effects of EMS, has an increased frequency of EMS-induced chromosomal aberrations, and is slow to repair DNA single-strand breaks compared to the parental CHO9 line (13). They found that the mutational spectra for EM-C11 and CHO9 had similar frequencies of GC-to-AT transitions, but in the EM-C11 cells, AT-to-GC transitions were replaced by a group of mutations (24%), resulting in exon exclusion (13). Op het Veld et al. (13) postulated that unrepaired single-strand breaks could be responsible for the generation of the deletion type of mutations. Our previous studies have provided evidence that nutritional folate deficiency induces DNA strand breaks in CHO cells and that the combination of EMS and folate deficiency results in many more breaks than either condition alone (4). Moreover, we found that repair of EMS-induced strand breaks was incomplete in low-folate medium compared to that in cells in folate-replete medium (4). These results are consistent with the work of Borchers et al. (15), who observed that methotrexate, a folate antagonist, caused significant accumulation of single-strand breaks in CHO cells exposed to EMS. Coadministration of hypoxanthine and thymidine in methotrexate-treated cells prevented single-strand break accumulation, suggesting that nucleotide depletion by methotrexate inhibited repair synthesis (15). A similar mechanism may explain the results reported here. EMS treatment could produce single-strand breaks that are unrepaired due to folate deficiency, and these breaks are responsible for deletions, whereas cells in folate-replete medium are able to repair most of the breaks and thereby manifest fewer deletions.

Gene deletions and related mutations, such as DNA translocations, are common in malignant tumors. It is of interest that molecular analysis of p53 tumor suppressor gene mutations in adenomatous polyposis coli identified deletions and insertions as the predominant mutations (16) because epidemiologic studies have identified folate deficiency as a risk factor for colorectal adenomas or cancer (6). Consistent loss of chromosomal regions has been observed in myelodysplastic syndromes and acute myelogenous leukemia (17). This is particularly frequent in therapy-related myelodysplasia and acute myelogenous leukemia associated with alkylating agents. These diseases usually manifest deletions of chromosomes 5 and 7 and, less commonly, deletions of 17p, 12p, and 20q (17). Although EMS is not used clinically, its effects on the genome resemble cyclophosphamide, a widely used drug well known for its association with treatment-related leukemias (18, 19). Our laboratory previously has reported that substantial increases in mutant frequency at the hprt locus occur in folate-deficient women who are treated with cyclophosphamide for breast cancer (20). Taken together, these observations support the idea that folate supplementation of deficient patients may decrease the frequency of intragenic deletions caused by some alkylating agents and thereby decrease the risk of developing secondary malignancies.

### Table 2

<table>
<thead>
<tr>
<th>Mutant frequency (× 10⁻⁶ ± SE)</th>
<th>Treatment</th>
<th>Folate-replete</th>
<th>Folate-deficient</th>
<th>Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>23 ± 1</td>
<td>9</td>
<td>38 ± 12</td>
<td>9</td>
</tr>
<tr>
<td>EMS</td>
<td>618 ± 61</td>
<td>3</td>
<td>1796 ± 165</td>
<td>3</td>
</tr>
<tr>
<td>ENU</td>
<td>838 ± 58</td>
<td>6</td>
<td>3070 ± 2151</td>
<td>6</td>
</tr>
</tbody>
</table>

* n = no. of independent experiments.

EMS has high nucleophilic selectivity and reacts predominantly with nitrogen atoms, characteristics which are associated with the induction of cytotoxicity and structural chromosomal aberrations (8, 9). In contrast, ENU has low nucleophilic selectivity and reacts with both nitrogen and oxygen atoms. The latter reaction results in miscoding lesions, leading to point mutations (8, 9). EMS produced about twice as many sister chromatid exchanges as ENU in CHO cells at doses yielding equal mutation frequencies (10) and exhibited higher mutagenic activity (13).

### Table 3

<table>
<thead>
<tr>
<th>Multiplex DNA amplification to detect deletions at the hprt locus in CHO cells</th>
<th>Treatment</th>
<th>No. of mutant clones analyzed</th>
<th>Deletions</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS</td>
<td>Folate-replete</td>
<td>16</td>
<td>None</td>
<td>0/16 (&lt;5.9%)</td>
</tr>
<tr>
<td>ENU</td>
<td>Folate-replete</td>
<td>26</td>
<td>Exon 9 (1)</td>
<td>1/26 (3.8%)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent number of clones.
References

Effect of Folate Deficiency on Mutations at the hprt Locus in Chinese Hamster Ovary Cells Exposed to Monofunctional Alkylating Agents
