Expression of the Biochemical Defect of Methionine Dependence in Fresh Patient Tumors in Primary Histoculture

Hui-Yan Guo, Hector Herrera, Ami Groce, and Robert M. Hoffman

Abstract

Methionine dependence is a metabolic defect that occurs in many human tumor cell lines but not normal in unestablished cell strains. Methionine-dependent tumor cell lines are unable to proliferate and arrest in the late S/G2 phase of the cell cycle when methionine is replaced by its immediate precursor homocysteine in the culture medium (MET-HCY\* medium). However, it is not known whether methionine dependence occurs in fresh patient tumors as it does in cell lines. In order to determine whether methionine dependence occurs in fresh patient tumors as well as in cell lines we took advantage of the technique of sponge-gel-supported histoculture to grow tumors directly from surgery. We then measured nuclear DNA content by image analysis to determine the cell cycle position in MET-HCY\* compared to MET-HCY\*-medium in 21 human patient tumors. Human tumor cell lines found to be methionine dependent by cell count were used as positive controls and were found to have marked reduction of cells in G1 compared to total cells in the cell cycle in MET-HCY\* medium with respect to the G1: total cell ratio in MET-HCY\*-medium. Therefore late cell cycle arrest was used as a marker of methionine dependence for histocultured patient tumors. We found that 5 human tumors of 21, including tumors of the colon, breast, ovary, prostate, and a melanoma, were methionine dependent based on cell cycle analysis. These data on fresh human tumors indicate that methionine dependence may frequently occur in the cancer patient population. Implications for potential therapy based on methionine dependence are discussed.

Introduction

Methionine dependence, the inability of tumor cells to grow in vitro when methionine is removed from the culture medium and replaced by homocysteine (MET-HCY\*)\* occurs frequently in malignant cell lines derived from all types of cancer (1-7). Normal cells and tissues which have been tested are methionine independent and still grow after methionine is replaced by homocysteine (1, 3, 8, 9). The question remains, however, whether methionine dependency also occurs in fresh human solid cancers and not just in established cell lines.

Under conditions of a limiting methionine source, methionine-dependent tumors arrest in the late S/G2 phase in the cell cycle (10). Methionine dependence may be due to overutilization of methionine for transmethylation reactions resulting in low free methionine pools and low S-adenosylmethionine:S-adenosylhomocysteine ratios, thereby blocking cell division under conditions of a limiting methionine source (11-13). In vitro treatment protocols were designed by us to exploit the methionine dependence of tumors with methionine starvation and cell-cycle-specific chemotherapy, such that in cultures of tumor and normal cells only the normal cells could survive (8).

Recently a number of investigators have attempted to exploit the methionine dependence of tumors for therapeutic effects in vivo. Breillout et al. (14) found for the RMS-J1 rat rhabdomyosarcoma tumor that a methionine-depleted diet lowered the metastatic potential of the tumor without significant effects on local tumor growth in rats (14). Goseki et al. (15) found that a methionine-free total parental nutrition mixture for rats bearing the Yoshida sarcoma extended the survival of the rats and slowed tumor growth in the rats, especially with the use of doxorubicin. Kreis and Hession (16), with the use of a methioninase, demonstrated an attenuation of growth of the W-256 rat carcinosarcoma growing in rats. We have demonstrated recently that the Yoshida tumor growing in nude mice can regress with prolonged dietary methionine starvation with an extended survival period of the mice compared to tumor-bearing mice on a methionine-containing diet.4

In this report we describe a new assay of methionine dependence that can be applied directly to freshly explanted human solid tumors in primary histoculture on collagen-containing gels. The assay determines the effects of methionine deprivation on the cell cycle by measuring DNA content with an image analyzer. The results indicate that methionine dependence indeed occurs in fresh human cancer in 5 of 21 patient cases analyzed. The implications for therapy are discussed.

Materials and Methods

Cell Culture. The following cell lines or strains were studied as controls for the study of fresh tumor surgical specimens: methionine-independent human foreskin fibroblast strain (FS-3) (negative control); methionine-dependent cell lines of prostate cancer PC3: lung carcinoma SKLU-1; and fibrosarcoma HT 1080 tumor cell lines (positive controls). The cell strain and lines have been previously demonstrated to be methionine independent or methionine dependent, respectively, by cell count after culture in MET-HCY\* compared to MET-HCY\*-media (1). The normal cell strain and tumor lines were cultured in Eagle's minimum essential medium with dialyzed fetal bovine serum which was methionine free. The medium was supplemented with 100 \( \mu \)M folic acid, 1.5 \( \mu \)M hydroxocobalamin, nonessential amino acids (1:100 dilution of a stock solution from Irvine Scientific), gentamicin at a concentration of 0.2 mg/ml, and either 100 \( \mu \)M L-methionine (MET-HCY\*-medium) or 200 \( \mu \)M L-homocysteine thiolactone (MET-HCY\*-medium). The high levels of folic acid and hydroxocobalamin were used in order to promote methionine biosynthesis in MET-HCY\*-medium (7). Cells and tissues were cultured in a gassed incubator with 95% sterile air/5% CO2.

Histoculture. Human patient tumors obtained at surgery were histocultured according to the technique described by Hoffman (17). Briefly, tissue was cut into 1-3-mm\* pieces which were then supported by collagen-containing sponge gels hydrated with culture medium as described above. The tumor tissues were histocultured in six-well dishes and incubated, as described above.

DNA Staining. The histocultured tumor tissue along with supporting gels were digested with 0.8% collagenase for 2 h at different time points of his...
Table 1  Cell cycle distribution ratios of human tumor cell lines and normal cell strain in MET* HCY* and MET* HCY* media

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G1/total cells in MET* HCY* medium</th>
<th>Methionine dependence by cell count (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human tumor</td>
<td>G1/total cells in MET* HCY* medium</td>
<td></td>
</tr>
<tr>
<td>PC-3 (prostate)</td>
<td>0.102</td>
<td>Dependent (1)</td>
</tr>
<tr>
<td>SKLU (lung)</td>
<td>0.562</td>
<td>Dependent (1)</td>
</tr>
<tr>
<td>HT1080 (sarcoma)</td>
<td>0.329</td>
<td>Dependent (1)</td>
</tr>
<tr>
<td>Average</td>
<td>0.33 ± 0.13</td>
<td>Independent (1)</td>
</tr>
<tr>
<td>Human normal</td>
<td>FS-3 (foreskin)</td>
<td>1</td>
</tr>
</tbody>
</table>

Results

Cell-Cycle Analysis of Normal Cell Strain and Malignant Cell Lines. The human foreskin fibroblast cell strain FS-3 was previously determined to be methionine independent by direct cell counting assays (1). No difference was seen in the ratio of cells in G1 to total cells as measured by DNA content after the cells were cultured in MET* HCY- medium or in MET* HCY+ medium for 21 days (Table 1). We have previously observed that normal human fibroblast strains do not change their cell cycle distribution value when grown in MET* HCY* versus MET* HCY+ media (10).

The human tumor cell lines shown in Table 1 were previously determined to be methionine dependent by cell count, by demonstrating their inability to grow in MET* HCY+ medium as opposed to MET* HCY- medium (1). These lines include prostate cancer PC3, lung carcinoma SKLU-1, and the sarcoma HT1080. When the DNA content of the cell nuclei was measured, these cell lines showed a MDCCB value (ratios of cells in the G1 phase of the cell cycle to total cells in the cycle in MET* HCY- medium:MET* HCY+ medium) of less than 0.65 (Table 1; Fig. 1). The results suggest that most of the tumor cells in MET* HCY+ medium replicated their DNA but could not complete mitosis and reenter the G1 phase in the cell cycle. The tumor cell lines were methionine dependent and thus unable to grow in MET* HCY+ medium as measured directly by cell count as mentioned above. In addition, unlike normal cells, the tumor cells lines accumulated many cells in the late phase of the cell cycle because they...
were unable to divide and enter G1 (1). Thus cell-cycle analysis was validated as an additional determinant or marker of methionine dependency.

Cell-Cycle Analysis of Histocultured Patient Tumors. Five of 21 human patient tumors were found to be methionine dependent and had a mean MDCCB value of 0.515 (Table 2, Group I). These tumors include colon cancer (patient ID no. 2518), melanoma (2140), ovarian cancer (2443), prostate (2137), and breast cancer (2468). Fig. 2 shows the cell cycle distribution of all the Group I tumors in MET+HCY− compared to MET+HCY+ media.

On the other hand, the Group II tumors of Table 2 have a mean MDCCB value of 0.97, indicating methionine independence. Fig. 3 shows the cell cycle distribution of representative Group II tumors in MET+HCY− compared to MET+HCY+ media. The difference between Group I and Group II tumors was significant, with a P value of 5.73 × 10−4. The P value comparing the cell cycle distribution (MDCCB) of Group I tumors of Table 2 to that of the methionine-dependent cell lines of Table 1 was 0.152, suggesting that Group I tumors are similar to the methionine-dependent cell lines and are therefore themselves methionine dependent. The P value comparing the cell cycle distribution of Group II tumors to the methionine-dependent cell lines was 4.89 × 10−4, suggesting that Group II tumors are different from the methionine-dependent tumor cell lines and are therefore methionine independent. This is further emphasized by their MDCCB ratio of approximately 1, as mentioned above.

When methionine-dependent patient tumor no. 2140 was shifted from MET+HCY− medium to MET+HCY+ medium, a normal cell cycle distribution resumed (data not shown), demonstrating the reversibility of the cell cycle block in the patient tumors. A similar reversibility was previously observed with malignant cell lines (10).

Discussion

The results presented in this report demonstrate that 3-dimensional histoculture and cell cycle analysis allow the determination of the methionine dependency of fresh human solid tumors and that indeed methionine dependence occurs in a sizable proportion of fresh human tumors. Thus 5 of 21 human tumors analyzed for cell cycle distribution in histoculture in MET+HCY− versus MET+HCY+ media were determined to be methionine dependent.

The strength of the analysis includes the fact that the human tumor cell lines used as standards for cell cycle analysis have been determined to be methionine dependent by cell count by their lack of growth in MET+HCY− medium as opposed to MET+HCY+ medium (1). These same tumor lines were found to have a cell cycle block in MET+HCY− as opposed to MET+HCY+ media, as measured by the G1/total cell count, determined by cellular DNA content.

We were able to grow patient tumors directly from surgery in histoculture over the long term with the use of collagen sponge gel supports. When the cell cycle analysis was applied to the histocultured
tumors it was assumed that a value of 1 for MDCCB would define methionine independence, based on our previous data using monolayer cultures of methionine-independent normal and methionine-dependent tumor cells (10). A normal distribution was assumed around the value of 1 for MDCCB. Eight cases had values somewhat over 1, and it was therefore assumed that 8 cases would have values under 1 as part of a normal distribution. The cutoff to distinguish methionine dependence and methionine independence was therefore assumed to

![Fig. 3. Cell cycle distribution of human patient tumors found to be methionine independent (Group II). Tumors were histocultured in MET- HCY medium or in MET+ HCY medium, stained with Schiff’s reagent, and measured for DNA content, as described in the text. Note lack of accumulation of cells with increments in DNA content in MET+ HCY medium for tumors in Group II, indicating methionine independence.](image-url)
be below this normal distribution, which was 0.65 for MDCCB. Using a cutoff of 0.65, Group I (methionine-dependent) and Group II (methionine independent) were different with a P value of 5.73 × 10⁻⁴, and Group I matched the methionine-dependent cell lines confirmed by cell count as well as cell cycle analysis. It may be possible that the method of analysis may underestimate the number of methionine-dependent tumors. By these criteria normal fibroblasts are methionine independent (Table I) (10).

The cell cycle block of the methionine-dependent tumors can be reversed by shifting from MET⁻ HCY⁺ to MET⁺ HCY⁻ media, as was found for melanoma tumor no. 2140 (data not shown).

Selectively synchronizing tumor cell growth is a potential means of enhancing the action of cell cycle-specific cytotoxic agents. Many agents have been reported to synchronize cell growth. For example, lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, arrests normal and tumor cells of epithelial as well as fibroblast origin reversibly in the G₁ phase of the cell cycle. This arrest could be reversed by the addition of mevalonic acid (the product of the reaction catalyzed by the 3-hydroxy-3-methylglutaryl-coenzyme A reductase) (18). It has been demonstrated that the combination of hormone and polyamine depletion/repletion yielded significant synchronization with a block in G₁ in N-nitrosomethylurea-induced mammary tumors in rats (19). Upon release of the block, the cells then synchronously entered S phase as measured by [³H]thymidine labeling indices. Hoechst 3342 (bisbenzimid H 33342 fluorochrome) and VM-26 (teniposide), inhibitors of DNA topoisomerase II, arrested Chinese hamster ovary cells and human diploid dermal fibroblast (HSF-5) reversibly in the G₂ phase (20). Aortic endothelial cells as well as tumors accumulated in the G₂ + M phase after 2–10 Gy of ⁶⁰Co γ-ray irradiation (21).

Our data suggest that the methionine-dependent tumor cells can be synchronized selectively, however, by the late S/G₂ arrest induced by methionine starvation (10). This is the only clear evidence, to our knowledge, of tumor-specific cell cycle synchronization, and this may have therapeutic ramifications for cell cycle-dependent chemotherapy.

In this light, recent in vivo studies of the effects of methionine depletion by both dietary⁴ and enzymatic means⁵ have indicated anti-tumor efficacy. Methionine depletion has also led to tumor cell cycle arrest in vivo.⁴

In summary, the results presented in this report indicate that fresh human solid tumors frequently express methionine dependence and suggest that methionine is a potential target for cancer chemotherapy.

References
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