Combination Cytotoxic-Differentiation Therapy of Mouse Erythroleukemia Cells with 5-Fluorouracil and Hexamethylene Bisacetamide

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ABSTRACT

The effects of 5-fluorouracil (5-FUra), in combination with various differentiation inducers on the growth and differentiation of mouse erythroleukemia (MEL) cells were investigated. The cells were first treated with 5-FUra, washed, and then treated with various concentrations of differentiation inducers: hexamethylene bisacetamide (HMBA), dimethyl sulfoxide (DMSO), and N-methylformamide. Pretreatment with 5-FUra, shown here to be a weak inducer of MEL cell differentiation, enhanced the subsequent HMBA induction of differentiation. The three inducers of differentiation markedly inhibited cell growth and increased cell death in a dose- and time-dependent manner if given immediately after cells were exposed to 5-FUra. In contrast, 5-FUra at similar concentrations inhibited cell growth, but only slightly increased cell death, while inducers without 5-FUra had little effect on cell growth or viability. When placed in fresh drug-free medium for 6 days following drug treatments, the cells completely recovered from the growth inhibition of 5-FUra as a single agent, whereas in cells previously treated with only HMBA there was an inhibition of cell growth without loss of viability. In contrast, a profound and prolonged growth inhibition with 98% cell death occurred in cells previously treated with 5-FUra followed by HMBA.

The enhancement of 5-FUra cytotoxicity appeared to be directly related to the degree of differentiation and to biochemical events that occur during the commitment to terminal cell division induced by N-methylformamide, DMSO, or HMBA. An increase in Okazaki fragments was found in MEL cells treated with HMBA or DMSO when committed to terminal cell division. DNA breaks also follow 5-FUra treatment (A. Yoshioka et al., J. Biol. Chem., 262:8235-8241, 1987) and may be the events that lead to cell death. The marked increase in cell death resulting from 5-FUra/HMBA treatment may be, at least partly, a consequence of increased DNA breaks due to 5-FUra followed by inhibition of DNA repair which is known to occur following the HMBA or DMSO induction of differentiation and commitment to terminal cell division. This combined sequential cytotoxic-differentiation therapy resulting in synergistic cytotoxicity and differentiation may be the basis of a new approach to cancer therapy and may aid in reducing the amounts of chemotherapeutic agents required for effective treatment, while maintaining or even increasing their therapeutic effects.

INTRODUCTION

5-Fluorouracil and its derivatives are widely used in cancer chemotherapy (1-4). However, the mechanism(s) of action of these compounds remains controversial. 5-FUra is converted to 5-FdUrd, which is an inhibitor of thymidylate synthase activity (1-4) and forms a ternary complex in the presence of equimolar concentrations of 5, 10-methylene tetrahydrofolate (a cofactor of the enzymatic reaction) (5, 6). The use of 5-FUra in combination with leucovorin, a reduced form of folate, has been shown to enhance the cytotoxic effect of 5-FUra (7-9) and to be of clinical benefit (10). An alternative mechanism by which 5-FUra might exert its cytotoxic action is by incorporation as 5-FdUr into cellular RNA (11, 12). However, since the cytotoxic effect of 5-FUra is significantly enhanced by leucovorin in cells that are folate deficient (13), it is probable that the primary mechanism responsible for the cytotoxicity of 5-FUra is due to inhibition of thymidylate synthase which can result in unbalanced deoxyribonucleotide triphosphate pools and presumably alter DNA metabolism (14, 15). It has been suggested that the DNA double-strand breaks which follow 5-FUra treatment are related to the “unbalanced pools” and may be the lethal event induced by 5-FUra (15). This effect of 5-FUra has yet to be exploited for maximal therapeutic effect.

DNA breaks are found in Friend leukemia virus-infected mouse erythroleukemia (MEL) cells when induced to differentiate and commit to terminal cell division by certain agents such as hexamethylene bisacetamide (HMBA) and dimethyl sulfoxide (DMSO) (16, 17). There is an irreversible reduction in DNA ligase activity (18) and an increase in calcium-, magnesium-dependent nuclear DNA endonuclease activity (19) in MEL cells committed to terminal cell division by these agents. MEL cells in specific phases of the cell cycle (i.e., S phase) have been reported to be more inducible to differentiate and commit to terminal cell division (20). In view of these findings it seemed reasonable to expose cells to 5-FUra, a drug that is cell cycle specific, to “stage” some cells into the S phase of the cell cycle to make them more responsive to HMBA induction of differentiation and commitment to terminal cell division. The predicted results would be HMBA-enhanced 5-FUra toxicity due to: (a) enhanced HMBA induction of differentiation and terminal cell division by pretreatment of the cells with 5-FUra; (b) enhanced 5-FUra-induced DNA breaks as a result of HMBA inhibition of DNA repair. It is this scheduled interaction that we call combined cytotoxic-differentiation therapy. We report here that MEL cells treated with 5-FUra followed by HMBA undergo a profound and prolonged inhibition of growth and a marked increase in cell death.

MATERIALS AND METHODS

Materials. 5-FUra, dexamethasone, and leucovorin (calcium) were obtained from SoloPak Laboratories, Franklin Park, IL, Sigma Chemical Co., St. Louis, MO, and Lederle Parenterels, Inc., Carolina, PR, respectively. 5-FUra was stored at room temperature. A stock solution was prepared daily by diluting 1:1000 in complete medium (medium containing 15% (v/v) fetal bovine serum, penicillin, and streptomycin as described (21). Dexamethasone, 1.95 mM, was prepared daily in ethanol and then a stock solution of 1 mM was prepared from this with complete medium. Media, serum, and other chemicals were obtained and MEL cell lines DS-19 and DR-10 (22) were grown as previously described (21, 23).

Cells. Cells in logarithmic growth were seeded at 2 x 10^5/ml into 3 ml prewarmed complete media with or without 5-FUra and leucovorin, as indicated in “Results” and the figure legends. Seven, 8, or 17 h later as indicated in the text and figure legends, the cells were collected by centrifugation at 275 x g and 4°C for 5 min, washed with 3 ml of...
complete medium, followed by an identical centrifugation, and the entire cell pellet was resuspended into 3 ml fresh, complete medium with and without an inducer (DMSO, hemin-C1, HMBA, or NMF) and with or without dexamethasone as indicated in the figure legends. The cells were harvested 4 days after initially adding 5-FUra (unless specifically noted) and then cell concentrations, the percentage of nonviable (trypan blue positive) cells, and the percentage of differentiated (hemoglobin-containing, benzidine-positive, B+) cells were determined as described (24).

Alkaline Sucrose Gradient Centrifugation. Cells were passed daily at 1.5 x 10^6 cells/ml complete medium with or without added inducers of differentiation such as 1.8% DMSO, 5 mM HMBA, or 200 µM hemin. At the times indicated, cells were harvested by centrifugation at 4°C and resuspended in the absence of inducers in 1 ml prewarmed Hank's balanced salt solution (GIBCO, Grand Island, NY), 15% (v/v) dialyzed fetal bovine serum (GIBCO) to a concentration of 5 x 10^5 cells/ml. [3H]Thymidine, 46 Ci/mmol (Amersham, Woburn, MA) at 40 µCi/ml was added for 1 min at 37°C. Reactions were stopped with 5 ml ice-cold phosphate-buffered saline (GIBCO). The cells were centrifuged to remove the supernatant solution and lysed by the addition of 1% (w/v) Sarcosyl NL30 (Gallard-Schlesinger Industries, Inc., New York, NY) in 0.45 M NaOH, 0.55 M NaCl, and 0.01 M EDTA. Alkaline sucrose gradient analysis was performed as previously described (25). Aliquots of 200 µl containing 4 x 10^5 cells were carefully layered over 5-20% (w/v) linear sucrose gradients in 0.1 M NaOH, 0.9 M NaCl, 0.01 M EDTA, and 0.015% Sarcosyl over a 60% (w/v) sucrose "cushion" solution and immediately centrifuged at 44,000 rpm in an SW50.1 rotor at 20°C for 3 h in a Beckman LC2B preparative ultracentrifuge. The gradients were collected on Whatman GF/A glass fiber filter disks as previously described (26) and were subjected to trichloroacetic acid precipitation followed by ethanol and ether washes; then the amount of radioactive material present on the disks was determined with the aid of a Beckman liquid scintillation spectrometer after placing the disks in Aquasol 2 (Du Pont-New England Nuclear, Boston, MA).

RESULTS

The Effects of 5-FUra and Differentiation Inducers on Growth and Differentiation of MEL Cells. Exposure of MEL cells to 5-FUra (2-12 µM) for 7-8 h led to a concentration-dependent decrease in cell number, reaching 50% of the yield of untreated cultures at approximately 11 µM (Fig. 1A) with only a slight increase in the percentage of nonviable cells (Fig. 1B). 5-FUra was found to be a weak inducer of differentiation (Fig. 1, C and D). Whereas untreated cultures contained from 0 to 0.3% hemoglobin-containing cells, after exposure to 12 µM 5-FUra, 10% of the cells were found to be B+ (Fig. 1C) and at higher 5-FUra concentrations up to 20% were B+ (Fig. 1C, also see Fig. 8C). However, apparently due to the cytotoxicity of 5-FUra, the number of B+ cells never exceeded 5% (Fig. 7D). When DMSO, NMF, or HMBA were added individually at optimal concentrations for inducing differentiation (see Refs. 27 and 28) they increased the cell number slightly (Fig. 4 legend) and only increased the percentage of nonviable cells from 4% in untreated cultures to about 7% (Fig. 1B). However, when these agents were added following 5-FUra treatment, the concentration of 5-FUra required to decrease the cell number to 50% was markedly reduced compared to that in cultures treated with 5-FUra alone (Fig. 1A), while more than doubling the percentage of nonviable cells in cultures treated with 12 µM 5-FUra alone (Fig. 1B). As expected, DMSO was a more potent inducer of differentiation than NMF (see Ref. 28) (Fig. 1C). Pretreatment with certain concentrations of 5-FUra further enhanced the percentage of B+ cells accumulated (Fig. 1C). However, at concentrations of 5-FUra greater than 8 µM this effect was not observed, apparently due to the decrease in cell growth and the high percentage of nonviable cells (Fig. 1, A and B). Since

![Fig. 1. Effect of 5-FUra (5-FU) treatment followed by exposure to polar-planar inducers on the growth and differentiation of MEL cells. Cells in logarithmic growth were treated and 5-FUra at the indicated concentrations for 7 h, then centrifuged as described in "Materials and Methods" and reseeded in fresh medium containing no inducers (○), 1% NMF (△), 1.8% DMSO (□), or 4 mM HMBA (○) for 88 h. The cell number (A), the percentage of nonviable cells (B), the level of differentiation (C) and (D) were determined as described in "Materials and Methods." The mean cell yields/ml in the absence of 5-FUra were 4.62 x 10^6 in noninducer-treated cultures, and 5.96 x 10^6, 5.34 x 10^6, and 4.9 x 10^6 in NMF-, DMSO-, and HMBA-treated cultures, respectively.](https://cancerres.aacrjournals.org/article/51/11/3879/0)
CYTOTOXIC-DIFFERENTIATION THERAPY OF MEL CELLS

Fig. 2. Effect of increased 5-FUra (5-FU) exposure followed by polar-planar-solvent inducers on the growth and differentiation of MEL cells. Cells in logarithmic growth were treated with 5-FUra for 17 h, then transferred to fresh medium without an inducer (•), or with 1% NMF (A), or 1.8% DMSO (△), and grown for 90 h. Then the cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D) were determined. The mean cell yields/ml in the absence of 5-FUra were 4.9 x 10⁶ in noninducer-treated cultures and 6.0 x 10⁶ and 5.9 x 10⁶ in NMF- and DMSO-treated cultures, respectively. The time course of HMBA enhancement of 5-FUra cytotoxicity is shown in Fig. 3. 5-FUra cytotoxicity was not enhanced when followed by an exposure to HMBA of less than 48 h; however, following longer exposures to HMBA, there was an enhancement of 5-FUra cytotoxicity (Fig. 3, A and B). For example, after 96 h of HMBA exposure following 5-FUra treatment, 85% of the cells were nonviable, whereas 5-FUra treatment alone led to only 10% of the cells becoming nonviable, which was similar to the level noted in untreated cultures (Fig. 3, A and B). The 5-FUra inhibition of cell growth was reversed within 48 h after the removal of 5-FUra, whereas in the presence of HMBA growth inhibition persisted (Fig. 3A).

due to DMSO or NMF at low, nontoxic 5-FUra concentrations, while at higher 5-FUra concentrations, no differentiation was observed (Fig. 2, C and D).

By using HMBA, an even more powerful differentiation inducer than DMSO, the concentration of 5-FUra required to decrease the cell yield 50% was reduced from approximately 14 μM 5-FUra to less than 6 μM, i.e., a greater than 50% reduction in the concentration of 5-FUra (Fig. 1A). The decrease in 5-FUra concentration required for reducing the cell number was combined with a dramatic increase in cell death when HMBA was included in the treatment regimen compared to the effect of 5-FUra treatment alone or to that of 5-FUra/DMSO treatment (Fig. 1B). HMBA was used at an optimal concentration for inducing differentiation (4 mM) (Fig. 1, C and D) that did not decrease cell yield (Fig. 1 legend) or increase the accumulation of nonviable cells (Fig. 1B). A 5-FUra concentration less than 8 μM slightly increased the HMBA induction of B⁺ cells, but a 5-FUra concentration greater than 8 μM decreased the ability of HMBA to induce differentiation, as well as the cell yield (Fig. 1D). On the other hand, treating the cells with HMBA (4 mM for 90 h) before 5-FUra treatment (8 μM for 7 h) did not enhance 5-FUra cytotoxicity (data not shown).

Fig. 3. Effect of time of exposure to HMBA following 5-FUra treatment on growth and differentiation. Cells in logarithmic growth were treated with 5-FUra (8 μM) for 7.5 h, washed, and placed in fresh medium with (○—○) or without (□—□) HMBA (4 mM) for 24–96 h. Cells not exposed to 5-FUra were similarly treated with (△—△) or without (□—□) HMBA. The cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D) were determined at the times indicated.
The induction of differentiation by HMBA was barely detectable at 24 h and then continued to increase with time (Fig. 3, C and D). Pretreatment of the cells with 5-FUra retarded the onset of differentiation of HMBA but at longer exposure to HMBA (i.e., 96 h) reached the same endpoint as HMBA alone at 40 h (Fig. 3C).

Four mM HMBA, although an optimal concentration for differentiation induction in vitro, is difficult to reach and maintain in a cancer patient (29, 30), so a concentration (1 mM) that is readily achievable without toxicity was tested. After exposure to 5-FUra for 7.5 h, 1 mM HMBA treatment did not dramatically decrease the cell number (Fig. 4A), but did more than double the percentage of nonviable cells that accumulated at the higher concentrations of 5-FUra tested (Fig. 4B). At this concentration of HMBA, very little differentiation induction occurred, although an apparent synergism in differentiation induction between HMBA and 10 μM 5-FUra was noted (Fig. 4, C and D).

Leucovorin Enhancement of 5-FUra/HMBA Cytotoxicity. Since 5-FUra followed by 1 mM HMBA was not more cytotoxic than 5-FUra alone, leucovorin was added to potentiate 5-FUra-induced cytotoxicity. Cells were pretreated with 5-FUra, or with 5-FUra plus leucovorin, and then with varying concentrations of HMBA. When leucovorin was added to the treatment regimen, the concentration of HMBA required to inhibit cell growth was markedly reduced (Fig. 5A). At 1 mM HMBA, leucovorin increased the percentage of nonviable cells nearly 10-fold over that seen in the absence of leucovorin. At HMBA concentrations greater than 2 mM, the addition of leucovorin did not enhance the reduction of cell number, but the percentage of nonviable cells was increased by leucovorin at all concentrations of HMBA tested (Fig. 5B). The addition of leucovorin inhibited the appearance of differentiated cells due to 5-FUra/
HMBA treatment (Fig. 5, C and D), apparently due to the increase in cytotoxicity.

Recovery from 5-FUra/HMBA Cytotoxic Treatment. Cells were grown with or without 8 μM 5-FUra for 8 h. Then they were collected by centrifugation, washed once in complete medium, and seeded at 2 × 10⁶ cells/ml in nonsupplemented medium or in medium containing 4 mM HMBA as described in “Materials and Methods.” After 90 h the cells were counted and reseeded in fresh medium without additives at a concentration of 920 viable cells/ml. The cultures were grown for 6 days. During this time the number of the cells in the untreated culture increased by 2580% (Table 1). The cells previously treated with 5-FUra increased their number similarly (2130%). Previous treatment with HMBA (4 mM) that induced 80% of the cells to differentiate (Fig. 1C) exhibited a subsequent inhibition of cell growth. This inhibition of cell growth was not characterized by an increase in dead (trypan blue positive) cells and probably represented cells induced to undergo terminal cell division by exposure to HMBA (31, 32). The growth of cultures previously exposed to 5-FUra/HMBA was severely inhibited with only a 15% increase in cell growth. In addition, 98% of the 5-FUra/HMBA-treated cells were nonviable compared to less than 10% of those in the untreated cultures.

Studies were then performed to determine whether the cells that “recovered” from 5-FUra/HMBA treatment were sensitive to a second cycle of treatment. Cells previously treated with 5-FUra/HMBA and fully “recovered” by 6 passages in fresh medium were normal in appearance and growth rate. Cells, growing in logarithmic phase, were then treated with various concentrations of 5-FUra alone or with 5-FUra followed by HMBA (4 mM) (Fig. 6). These cells reached approximately the same level of differentiation following HMBA treatment as did cells that had not been previously treated with 5-FUra/HMBA (Fig. 6, C and D). These cells became less sensitive to 5-FUra cytotoxicity since 14 μM 5-FUra no longer significantly inhibited cell growth or increased the level of nonviable cells (Fig. 6, A and B) as it had done in previously untreated cells (Fig. 1, A and B). However, treatment with 14 μM 5-FUra followed by HMBA still engendered as potent an inhibition of cell growth with greater than 95% loss of viability (Fig. 6, A and B) as it did following the original treatment (Fig. 1). These data suggest that previous exposure to 5-FUra followed by HMBA did not result in a rapid emergence of resistance to 5-FUra/HMBA, but perhaps exposure to 5-FUra in the absence of HMBA may have lead to resistance to 5-FUra.

Enhancement of 5-FUra Cytotoxicity by HMBA Appeared to be Related to Induction of Differentiation and/or Terminal Cell Division. To test the concept that the enhancement of 5-FUra cytotoxicity by HMBA or DMSO was related to the induction of differentiation, studies were done in DR-10, a variant MEL cell line that is resistant to DMSO-induced differentiation, but responsive to that by HMBA (Fig. 7, C and D) (22). The 5-FUra cytotoxic effect on DR-10 cells (Fig. 7B) was similar to its toxic effect on DS-19 cells (Fig. 1B), the parent line, with 50% of the cells rendered nonviable at approximately 13 μM 5-FUra. The cytotoxic effect of 5-FUra was not enhanced by DMSO in DR-10 cells, but it was by HMBA (Fig. 7, A and B). HMBA treatment reduced the concentration of 5-FUra required to decrease the cell number by 50% from approximately 8 to 4 μM and the concentration of 5-FUra required to render 50% of the cells nonviable from approximately 13 to 6 μM. Therefore, it appears that the induction of differentiation was needed for the potentiation of 5-FUra cytotoxicity.

In order to learn if commitment to terminal cell division might also be related to the potentiation of 5-FUra cytotoxicity by HMBA, the effect of hemin on 5-FUra cytotoxicity was determined (Fig. 8). Hemin is an inducer of MEL cell differentiation (33) that does not commit the cells to terminal cell division (34). Cells were treated with various concentrations of 5-FUra for 7.5 h, washed, and then treated with hemin (200 μM). Hemin treatment alone for 90 h induced over 20% of the cells to become B⁺ and differentiation was enhanced by 5-FUra

Table 1 Recovery of MEL cells from previous exposure to 5-FUra and/or HMBA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells/ml × 10⁶</th>
<th>Nonviable cells (%)</th>
<th>Increase in viable cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>258</td>
<td>8</td>
<td>2580</td>
</tr>
<tr>
<td>5-FUra</td>
<td>21.3</td>
<td>8</td>
<td>2130</td>
</tr>
<tr>
<td>HMBA</td>
<td>8.5</td>
<td>5</td>
<td>880</td>
</tr>
<tr>
<td>5-FUra + HMBA</td>
<td>0.77</td>
<td>98</td>
<td>15</td>
</tr>
</tbody>
</table>

Fig. 6. Effects of 5-FUra (5-FU) or 5-FUra/HMBA on growth and differentiation of cells previously exposed to 5-FUra/HMBA. Cells growing in logarithmic phase that had previously been treated with 5-FUra/HMBA (as in Table 1) and then “recovered” by 6 passages in fresh medium over a 3-week period were treated for 7 h with various concentrations of 5-FUra as indicated, followed by growth in fresh medium for 96 h without 5-FUra and in the absence (0) or presence of 4 mM HMBA (C) for 96 h. Then the cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D) were determined. The mean cell yields/ml in the absence of 5-FUra were 4.4 × 10⁶ in the absence of HMBA and 5.0 × 10⁶ in the presence of HMBA.
Fig. 7. Effects of DMSO and HMBA treatment following exposure to 5-FUra (5-FU) on growth and differentiation of DMSO differentiation-resistant MEL cells. DMSO differentiation-resistant (line DR-10) cells in logarithmic growth were treated with 5-FUra for 7 h, then transferred to fresh medium without further supplements (○), or with 1.8% DMSO (△), or 6 mM HMBA (□), and grown for 96 h. Then the cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D), were determined. The mean cell yields/ml in the absence of 5-FUra were 4.1 x 10^6 in noninduced cultures, and 3.9 x 10^6 and 4.1 x 10^6 in DMSO- and HMBA-treated cultures, respectively.

Fig. 8. Effect of 5-FUra (5-FU) treatment followed by hemin treatment on the growth and differentiation of MEL cells. Cells in logarithmic growth were treated with 5-FUra for 7.5 h, then transferred to fresh medium without supplements (○) or with hemin-CI (200 μM) (□) and grown for 90 h. Then the cell number (A), percentage of nonviable cells (B), the level of differentiation (C and D), and the expected level of differentiated cells (×) were determined. The expected value is the sum of the values from cultures treated only with hemin and those treated only with 5-FUra. The mean cell yields/ml in the absence of 5-FUra and in the absence and presence of hemin were 5.1 x 10^6 and 4.5 x 10^6, respectively.

The combination of 5-FUra and hemin only slightly increased cytotoxicity as compared to 5-FUra alone (Fig. 8, C and D). The concentration of 5-FUra required to obtain a 50% reduction in cell number was only decreased by hemin from 12 μM to approximately 11.5 μM 5-FUra (Fig. 8A), and hemin increased the level of nonviable cells by only 8 and 18% at 4 and 6 μM 5-FUra, respectively (Fig. 8B). At the highest concentration of 5-FUra tested, the reduction in cell number and the increase in nonviable cells were the same in the presence or absence of hemin. With a combination of 5-FUra and hemin that induced over 60% differentiation there was less than 20% cell death. The inability of hemin to enhance 5-FUra cytotoxicity despite its ability to induce differentiation supports the concept that commitment to terminal cell division may be required to enhance cytotoxicity.

Effect of Dexamethasone on HMBA Enhancement of 5-FUra Cytotoxicity. Dexamethasone inhibits induction of differentiation of MEL cells by DMSO (35, 36) and HMBA (37), as well as commitment to terminal cell division (37-39). However, dexamethasone does not appear to inhibit all of the features induced by these agents. Upon removal of dexamethasone and in the presence of an inducer, commitment to terminal cell division occurs much more rapidly than in cultures that are treated continually with an inducer with no exposure to dexamethasone (37-40). Studies were undertaken to evaluate the effect of dexamethasone on the HMBA potentiation of 5-FUra-induced cytotoxicity. Cells were treated with various concentrations of 5-FUra followed by HMBA, dexamethasone, or HMBA plus dexamethasone (Fig. 9). As expected, dexamethasone inhibited HMBA-induced differentiation following pretreatment.
cells (B), and the level of differentiation (C and D) were determined. The mean cell yields/ml in the absence of 5-FUra were 5.4 x 10^6 in noninduced cultures, dexamethasone (O O). Then the cell number (A), the percentage of nonviable medium and grown for 90 h without supplements (•—•), or supplemented with logarithmic growth were treated with 5-FUra for 7 h, then transferred to fresh medium and grown for 90 h without supplements (O O), or supplemented with HMBA (4 mM) (○—○), dexamethasone (30 μM) (●—●), or HMBA plus dexamethasone (□—□). Then the cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D) were determined. The mean cell yields/ml in the absence of 5-FUra were 5.4 x 10^6 in noninduced cultures, and 5.8 x 10^6, 4.4 x 10^6, and 4.9 x 10^6 in cultures treated with HMBA, dexamethasone, and HMBA plus dexamethasone, respectively.

with 5-FUra (Fig. 9, C and D). However, dexamethasone did not block the reduction in cell number due to 5-FUra/HMBA treatment (Fig. 9A), nor did it substantially inhibit the increase in nonviable cells seen following 5-FUra/HMBA treatment (Fig. 9B). In addition, dexamethasone did not inhibit the increase in nonviable cells due to treatment with 5-FUra alone (Fig. 9B), and only enhanced the reduction in cell number due to treatment with 5-FUra alone at the higher concentrations of 5-FUra tested (Fig. 9A).

Inhibition of DNA Formation and Repair by Differentiation Inducers. DNA ligase activity is significantly inhibited (18) and a calcium-, magnesium-dependent nuclear DNA endonuclease activity is markedly increased (19) in MEL cells induced to differentiate by HMBA. Earlier work also indicated that there was a time-dependent increase in the levels of small DNA species (Okazaki fragments) measured during the induction of differentiation by HMBA (28). The increased levels of Okazaki fragments were first measurable at 48 h after initiating induction and then they gradually increased in amount throughout the 96-h course of study. To further study the effect of various inducers on cellular DNA metabolism, cells were induced to differentiate with HMBA, DMSO, or hemin, harvested after 96 h of treatment, pulsed with high specific activity [3H]thymidine, and placed on an alkaline sucrose gradient (see “Materials and Methods”). After 96 h of treatment, cells treated with either HMBA or DMSO contained an increased level of Okazaki fragments as compared to untreated control cells (Fig. 10, A and B). The effect of DMSO, like that of HMBA, was time dependent, but the effects were not noted until 72 h of treatment. Hemin also induced a small increase in the level of Okazaki fragments, but much less than that induced by HMBA or DMSO (Fig. 10C; Table 2). “Pulse-chase” experiments carried out in untreated and DMSO-treated cells indicated that the radioactive material present in the low-molecular-weight fractions after a 1-min pulse were translocated into material present in the high-molecular-weight fractions after a 10 min chase. Thus the low-molecular-weight “Okazaki-like” fractions appeared to behave as precursors of the material of high molecular weight and not as breakdown products of it. Therefore commitment to terminal cell division as a consequence of HMBA- and DMSO-induced differentiation, appears to be associated with a defect in DNA propagation.

DISCUSSION

We have demonstrated a marked inhibition of MEL cell growth following exposure to 5-FUra and differentiation inducers. MEL cells are useful in studying the mechanism of the 5-FUra/HMBA interaction because of the well-defined stages of HMBA-induced differentiation and terminal cell division which can be probed separately by the use of various inducers and inhibitors of differentiation in native and variant cell lines. 5-FUra caused reversible cell growth inhibition without death (Fig. 1, A and B) unless prolonged or high 5-FUra concentrations were used (Fig. 2, A and B). In contrast, HMBA treatment for the same length of time, 96 h, did not inhibit cell growth (Fig. 1, A and B). However, when cells were exposed to a concentration of 5-FUra that produced reversible growth inhibition, were washed, and then treated with HMBA for 90 h at a concentration that is optimal for differentiation induction without cell growth inhibition, there was a profound loss of cell viability (Fig. 1, A and B). This enhancement of 5-FUra cytotoxicity required that cells first were treated with 5-FUra and then followed by HMBA treatment for 90 h rather than the reverse order. The enhanced toxicity appeared to be related to the induction of differentiation and commitment to terminal cell division. The enhancement of 5-FUra-induced cell death was directly related to the percentage of cells induced to differentiate (Fig. 1), to the time of inducer exposure (Fig. 3), and to the concentration of inducer used (Fig. 5). 5-FUra cytotoxicity was not increased by DMSO in a variant MEL cell line resistant to differentiation induction by DMSO but was increased by HMBA which induces differentiation in this line (Fig. 7), further supporting the process of differentiation in this cytotoxic effect. 5-FUra may enhance HMBA induction of differentiation by perturbing the cell cycle (48) of treated cells, thereby making them more responsive to HMBA induction of differentiation (20). 5-FUra at some concentrations was found to be a weak

Fig. 9. Effect of dexamethasone on 5-FUra (5-FU)/HMBA toxicity. Cells in logarithmic growth were treated with 5-FUra for 7 h, then transferred to fresh medium and grown for 90 h without supplements (O O), or supplemented with HMBA (4 mM) (○—○), dexamethasone (30 μM) (●—●), or HMBA plus dexamethasone (□—□). Then the cell number (A), the percentage of nonviable cells (B), and the level of differentiation (C and D) were determined. The mean cell yields/ml in the absence of 5-FUra were 5.4 x 10^6 in noninduced cultures, and 5.8 x 10^6, 4.4 x 10^6, and 4.9 x 10^6 in cultures treated with HMBA, dexamethasone, and HMBA plus dexamethasone, respectively.

B. Scher, unpublished results.
The position of an SV40 Form I marker (53S) is indicated by the arrow.

cells/ml medium in the absence (•—•) or in the presence (O O) of (A) 5 mM (Fig. 4) or hemin (Fig. 8). However, the 5-FUra augmentation differentiation by suboptimal inducing concentrations of HMBA
combined with induction of differentiation and commitment to terminal cell division occurs during a period when tumor cells are recovering from 5-FUra cytotoxicity; a time in cancer treatment that is often left drug free.

The optimal combination therapy with 5-FUra and HMBA appeared to require a concentration of 5-FUra that caused reversible cell growth inhibition followed by a concentration of HMBA that is optimal for differentiation induction. Although an optimal differentiation concentration of HMBA (4 mM) can be obtained in patients for prolonged periods of time, this is associated with metabolic acidosis and neurotoxicity (29, 30).

Our studies demonstrated that cytotoxicity produced by 5-FUra given with leucovorin was enhanced by a lower, clinically achievable, concentration of HMBA (1 mM) (Fig. 5). In these studies, 90% of the cells became nonviable when treated with 5-FUra (8 μM) and leucovorin (10 μM) for 7.5 h followed by 1 mM HMBA for 90 h (Fig. 5). The 5-FUra/leucovorin/HMBA combination produced irreversible cytotoxic effects, whereas 5-FUra/leucovorin given at similar doses produced only reversible cytotoxicity in MEL cells (9). Cytotoxicity produced by increased time of exposure to 5-FUra was not significantly enhanced by HMBA (Fig. 2). These observations suggest that the HMBA enhancement of 5-FUra/leucovorin cytotoxicity may be related to the irreversible inhibition of thymidylate synthase by 5-FUra in the presence of leucovorin followed by a defect in DNA metabolism that is potentiated by HMBA.

Differentiation induction and commitment to terminal cell division in MEL cells can be biologically dissected by using specific inducers and inhibitors of differentiation (41). The observation that 5-FUra cytotoxicity is not markedly enhanced by hemin (Fig. 8), a differentiation inducer that does not commit cells to terminal cell division (34), suggests that induction of terminal cell division is required for the enhanced cytotoxicity. Dexamethasone is an inhibitor of HMBA induction of differentiation in MEL cells but DNA hypersensitive sites (which are thought to be related to differentiation induction) and a "memory" for an increased rate of induction of terminal cell division are retained during dexamethasone treatment (37–40, 42). However, while dexamethasone inhibits HMBA differentiation induction (Refs. 35–37; Fig. 9, C and D), it did not inhibit HMBA enhancement of 5-FUra cytotoxicity (Fig. 9, A and B). These data are consistent with the concept that there are dexamethasone-resistant events that are related to the induction of differentiation, and occur during the commitment to

<table>
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<th>Material sedimenting at</th>
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</tr>
<tr>
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<td>20.4</td>
<td>20.6</td>
</tr>
<tr>
<td>Hemin</td>
<td>25.5</td>
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</tr>
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</table>

Table 2. Size distribution of [3H]thymidine-labeled acid-precipitable material

Radiolabeled thymidine was incorporated into acid-precipitable material and analyzed by alkaline sucrose gradient centrifugation as described in "Materials and Methods." Cells were grown for 96 h in the absence and presence of 1.8% (v/v) DMSO, 5 mM HMBA, and 200 mM hemin. Incubation of cell cultures with these compounds resulted in accumulations of B+ cells of 96, 96, and 70%, respectively, while the untreated control cultures accumulated less than 1% B+ cells. The data are taken from Fig. 10.

HMBA would not enhance 5-FUra cytotoxicity in normal proliferating cells, thereby giving the 5-FUra/HMBA combination possible tumor cell selectivity. In addition, the inhibition of tumor cell growth resulting from HMBA-induced terminal cell division occurs during a period when tumor cells are recovering from 5-FUra cytotoxicity; a time in cancer treatment that is often left drug free.

Fig. 10. Alkaline sucrose gradient centrifugation profiles of intermediates of DNA metabolism in lysates prepared from cells subcultured daily at 1.5 × 10⁶ cells/ml medium in the absence (●—●) or in the presence (○○) of 5 mM HMBA, (●), 1.8% (v/v) DMSO (C), or 200 μM hemin. After 96 h of growth, cells were harvested, incubated for 1 min with [3H]thymidine, lysed, and subjected to alkaline sucrose gradient analysis as described in "Materials and Methods." The position of an SV40 Form I marker (53S) is indicated by the arrow.

inducer of MEL cell differentiation and also enhanced differentiation by suboptimal inducing concentrations of HMBA (Fig. 4) or hemin (Fig. 8). However, the 5-FUra augmentation of HMBA induction of differentiation was difficult to assess at higher concentrations of 5-FUra because of the marked decrease in cell number (Fig. 2, C and D).

5-FUra/HMBA treatment of MEL cells resulted in prolonged cell growth inhibition and 98% loss of viability despite 6 subsequent days of culture in drug-free medium (Table 1). This may be due to HMBA-enhanced 5-FUra cytotoxicity combined with induction of differentiation and commitment to terminal cell division (Fig. 1C). The minimal HMBA toxicity in normal tissues found in clinical studies (29, 30) suggests that
terminal cell division that may be important in the HMBA potentiation of 5-FUra-induced cytotoxicity.

The time course of both HMBA-induced commitment to terminal cell division in MEL cells (32) and the HMBA-enhanced 5-FUra cytotoxicity (Fig. 3) were similar. Both were detectable after 48 h of HMBA exposure and increased with time. Commitment to terminal cell division is associated with a decrease in DNA ligase activity (18), an increase in a calcium-, magnesium-dependent nuclear DNA endonuclease activity (19), an increase in Okazaki fragments (Fig. 10; Table 2), and changes in DNA structure suggesting defective DNA repair (16, 17). Inhibition of DNA ligase alone is not sufficient to commit to terminal cell division since hemin inhibits DNA ligase activity (43) but does not significantly increase the level of Okazaki fragments (Table 2) or commit cells to terminal cell division (34). Therefore, certain changes in DNA metabolites associated with HMBA treatment, but not necessarily with hemin treatment, may be responsible for enhancing 5-FUra cytotoxicity.

DNA double-strand breaks are found in cells deficient in thymidylate synthase (44) and following exposure to high concentrations of 5-FdUrd (15). It has been suggested that DNA double-strand breaks accumulate as a consequence of the unbalanced deoxyribonucleotide pools due to 5-FUra or 5-FdUrd treatment and appear to be associated with the activity of a new nuclease (15). We suggest that the HMBA enhancement of 5-FUra cytotoxicity is associated with HMBA inhibition of DNA ligase activity and DNA repair, and that the cells become nonviable due to persistent 5-FUra-induced DNA single- and double-strand breaks. Further studies are required to clarify the mechanism(s) of the HMBA enhancement of 5-FUra cytotoxicity in this system such as direct measurement of DNA double-strand breaks following treatment with these agents alone or in combination. Additional possible explanations for the HMBA-enhanced 5-FUra cytotoxicity include a differentiation-induced down-regulation of transport of exogenous bases such as thymidine, and a decrease in the activities of enzymes that are ordinarily induced for increasing salvage and de novo pyrimidine synthesis to overcome 5-FUra cytotoxicity (45). Down-regulation of other transport systems by HMBA may also explain our observation that HMBA pretreatment followed by 5-FUra did not enhance 5-FUra cytotoxicity. Unlike NMF or DMSO (46–48), HMBA is probably not a free radical scavenger and would suggest that this is not the mechanism for the differentiation-inducer enhancement of 5-FUra cytotoxicity.

The scheduling of cytotoxic chemotherapy followed by differentiation inducers has been suggested previously (49, 50), but the role of differentiation has been, perhaps, best demonstrated by the use of 5-FUra/HMBA here. The enhancement of a cytotoxic agent by a differentiation inducer is not confined to MEL cells. A similar enhancement of 5-FUra by NMF has been observed in human colon carcinoma cells in vitro (51) and in vivo (52). Prolonged treatment of HT 29 colon carcinoma cells with HMBA irreversibly induces colonocyte differentiation (53). NMF, dimethylformamide, and DMSO enhance radiation antitumor effects in several tumor systems (54–56). cis-Diaminedichloroplatinum cytotoxicity is significantly enhanced when followed by NMF in mammary carcinoma cells both in vitro and in vivo (57). These observations suggest that differentiation inducers such as polar-planar compounds might be effective as additional chemotherapeutic agents when used following treatment with cytotoxic agents for various forms of cancer. Perhaps the emergence of resistance to cytotoxic agents can be diminished by combinations with differentiation inducers (49, 50). We present preliminary evidence that MEL cells that recover from treatment with 5-FUra/HMBA appear to be equally responsive to another course of 5-FUra/HMBA but less responsive to 5-FUra alone (Fig. 6). Although this observation needs further exploration, it suggests that HMBA altered cell metabolism, perhaps through induction of differentiation, in such a way that acquired 5-FUra resistance was diminished.

In summary, 5-FUra/HMBA produced irreversible MEL cell cytotoxicity at concentrations of each agent that are achievable in the clinic. There are interactions between these two agents that appear to promote mutual enhancement of cytotoxicity, i.e., 5-FUra is a weak inducer of MEL cell differentiation, 5-FUra enhanced the subsequent HMBA induction of differentiation, and HMBA potentiated the cytotoxic effect of 5-FUra. On the basis of these interactions, scheduling of a cytotoxic drug and a differentiation inducer may play an important role in cancer treatment. The use of a cytotoxic agent with a differentiation inducer we termed combination cytotoxic-differentiation therapy. Combination cytotoxic-differentiation therapy may lead to reductions in the effective amounts of certain chemotherapeutic agents, while increasing their therapeutic effectiveness by improving selectivity and decreasing resistance.

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REFERENCES


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Samuel Waxman, Barbara M. Scher, Nella Hellinger, et al.


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