Effect of Methylprednisolone on the Cell Kinetic Response of C3H/HeJ Mammary Tumors to Cyclophosphamide and Adriamycin

Paul G. Braunschweiger and Lewis M. Schiffer

Cancer Research Unit, Clinical Radiation Therapy Research Center, Division of Radiation Oncology, Allegheny General Hospital, Pittsburgh, Pennsylvania 15212

ABSTRACT

The effect of methylprednisolone (MP) on the cell kinetic response to cyclophosphamide (CP) and Adriamycin (ADR) in C3H/HeJ spontaneous mammary tumors and hematopoietic tissue was investigated. The [3H]deoxythymidine labeling index, the primer-dependent DNA polymerase labeling index (an estimate of tumor growth fraction), and the mitotic index were determined at various intervals after treatment. Treatment consisted of CP (200 mg/kg) on Day 0 plus ADR (2 mg/kg) on Day 1 with or without MP every 12 hr for 9 doses beginning on Day 2. In tumors treated with CP and ADR alone, changes in the kinetic parameters suggested proliferative recovery between Days 3 and 4 which coincided with bone marrow recovery. In tumors treated with CP, ADR, and MP, although the timing of the hematopoietic recovery was not affected by MP, the overshoot of the [3H]deoxythymidine labeling index on Days 3 and 4 was abolished. Proliferative recovery in the tumor was delayed until after cessation of MP treatments. Cell kinetic changes in the tumor after CP, ADR, and MP were used to design effective sequential chemotherapy which minimized the hematopoietic toxicity associated with sequential therapy designed from cell kinetic changes after CP and ADR alone.

INTRODUCTION

CP, ADR, and corticosteroids are 3 of the most widely used cancer chemotherapy agents. They have been used alone, but they are generally more effective when used in combination. Previous studies in animal systems have indicated that the cell kinetic perturbations induced by these agents can be useful for the design of sequential chemotherapy strategies (6, 8, 9).

Utilizing in vitro assay procedures to determine the [3H]dThd LI, the DNA synthesis time, and an estimate of tumor growth fraction by the PDP assay, we have studied the perturbing effects of MP, a synthetic glucocorticosteroid, on the cell kinetics of C3H/HeJ spontaneous mammary tumors (9). In these studies, we demonstrated G1 progression delay with synchronous progression through S phase after cessation of MP treatments. Treatment with cycle stage-specific agents was most effective when they were given at times when changes in the [3H]dThd LI indicated that most of the proliferating cells were in S phase.

The base-line cell kinetics of spontaneous mammary tumors in C3H/HeJ mice has been characterized (3, 20, 21), and previous studies have indicated that recovery of cell proliferation after CP and ADR occurs in this system between Days 3 and 4 (6), a time when bone marrow proliferation and repopulation may preclude institution of sequential therapy. The present study was designed to investigate the effects of MP on the cell kinetic response of C3H/HeJ spontaneous mammary tumors to CP and ADR.

MATERIALS AND METHODS

Animals and Tumors. Spontaneous mammary tumors arising in C3H/HeJ retired breeders were used in these experiments. Tumor-bearing mice were culled weekly from our retired breeder stock, housed 5 animals/cage, and fed standard laboratory mouse chow and water ad libitum.

Tumor-bearing mice were maintained on a 12-hr light-dark cycle and initiated into various studies when tumor volumes were approximately 0.5 cu cm.

Drug Treatments. Tumor-bearing mice were weighed and given drugs at the following dose levels: CP, 200 mg/kg; ADR, 2 mg/kg; MP, 10 mg/kg given every 12 hr for 9 doses; FUra, 42 mg/kg; and MTX, 6 mg/kg. In most studies, the following schedule was used: CP on Day 0 plus ADR on Day 1 with or without MP given every 12 hr for 9 doses beginning on Day 2. In some studies, MTX and FUra were administered at various times after CP and ADR. The interval between MTX and FUra administration was less than 15 min. Drug dosages were selected on the basis of toxicity considerations and to be consistent with previous studies (6, 9).

Cell Kinetic Measurements. The in vitro methods for the determination of the [3H]dThd LI have been described previously (2, 3). Briefly, tumor-bearing mice were killed by cervical dislocation, the tumors were quickly resected, and approximately one-half of the tumor was minced in McCoy’s medium supplemented with fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The remaining tumor tissue was fixed in neutral-buffered formalin and processed for histological sections. The tumor cell suspensions were labeled with [3H]dThd (15 to 17 Ci/mmol, 3 μCi/ml) for 30 min. In vitro labeling was done at 37° in a shaking water bath. Following the labeling interval, trypan-negative cells were separated by Ficoll-Hyphaque gradient centrifugation. Cells were fixed in Clarke’s fixative, and “drop” preparations were made for autoradiographic analysis as previously described. Autoradiographs were exposed for 1 or 2 days and developed by gold activation autoradiography as described previously (2). Background labeling was usually less than 1 grain per equivalent cell area, determined in areas adjacent to labeled cells. The grain count threshold was 3 grains per cell. Mean grain counts were usually in excess of 50 grains per labeled nuclei.

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1 To whom requests for reprints should be addressed, at Cancer Research Unit, Division of Radiation Oncology, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, Pa. 15212.

2 The abbreviations used are: CP, cyclophosphamide; ADR, Adriamycin; dThd, deoxythymidine; LI, labeling index; PDP, primer-dependent DNA polymerase; MP, methylprednisolone; FUra, 5-fluorouracil; MTX, methotrexate; M1, mitotic index; PDPI, primer-dependent DNA polymerase labeling index.

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The PDP assay is an autoradiographic method to measure the simultaneous presence of nuclear DNA polymerase α (5) and nuclear DNA primer template activity in individual cell nuclei. This technique has been described elsewhere in detail (5, 22, 26). Briefly, imprints from a freshly cut tumor surface were made on microscope slides and air-dried. The cytoplasm was stripped from the cells by dipping in 0.125% agar solution at 40°C and air-dried. The slides were then fitted with a glass ring incubation chamber into which is added 0.5 ml of an incubation mixture containing dATP, dGTP, and dCTP (0.2 mM), 5 mM MgCl₂, Ficol (Sigma Chemical Co., St. Louis, Mo.), and 10 µCi of [³H]dTTP per ml (specific activity, 55 to 60 Ci/mmol; New England Nuclear, Boston, Mass.) in 0.02 M Tris buffer, pH 7.4. The preparations were incubated at 37°C for 45 min, at which time the reaction was stopped on ice. The incubation rings were removed, and the slides were rinsed and fixed in acidic formaldehyde. The slides were subsequently rinsed again in deionized water prior to autoradiographic analysis. Autoradiograms were exposed for 7 to 10 days, and the fraction of labeled cells, determined from at least 500 cells, was designated the PDP index. Previous studies have shown that the PDP index is a good estimate of the growth fraction in unperturbed experimental tumor systems (3, 6, 26) and a responding modality after perturbation with drugs (6-9, 24, 25) and radiation (4). MI’s were determined from the paraffin sections by counting at least 4000 cells/tumor.

The [³H]dThd LI of femoral bone marrow and spleen cells from age-matched non-tumor-bearing controls was determined by a 30-min in vivo pulse labeling with [³H]dThd (2 µCi/g body weight; specific activity, 15 to 17 Ci/mmol), given i.p. Femoral marrow was obtained by flushing the diaphysial medullary cavity with 1 ml of fetal calf serum using a 1-ml tuberculin syringe. Marrow clumps were disaggregated by aspiration of the suspension with the tuberculin syringe and a 25-gauge needle. Spleen cells were obtained by mincing approximately 50% of the spleen in McCoy’s medium supplemented with fetal calf serum and filtering the resultant suspension through a nylon mesh to remove large clumps. After appropriate dilutions of marrow and spleen cells, and cytocentrifuge preparations were made. The cells were fixed in methanol, rinsed, and autoradiographed. Autoradiograms were developed after 2 weeks of exposure and stained by an acidified Giemsa method. LI’s were determined by evaluating at least 500 cells. In all counts, segmented polymorphonuclear leukocytes and late normoblasts were disregarded. Student’s t test was used for statistical analysis of the data.

RESULTS

Chart 1 shows the effect of ADR (2 mg/kg) and CP (200 mg/kg/12 hr for 9 doses) on the LI, MI, and PDPI in C3H/HeJ spontaneous mammary tumors. Each point represents the mean ± S.E. for at least 4 tumors.

The PDPI was significantly subnormal as compared to that in tumors treated with CP and ADR alone. No significant change was noted for the PDPI between 6 and 8 days after MP when both the LI and MI indicated a synchronous movement of cells through S phase.

Chart 2 shows the response of the [³H]dThd LI of maturing and dividing bone marrow cells to CP and ADR with and without MP. Without MP, recovery after dramatic LI reduction was initiated between Days 2 and 3 with maximal LI’s seen on Day 4. Normal LI’s were noted between Days 5 and 10 after treatment. Although MP treatment did not effect the onset of recovery after CP and ADR, the compensatory hyperproliferation seen at 4 days after CP and ADR alone was abolished by MP treatments. Normal or slightly subnormal LI’s were noted between Days 5 and 10 after CP.

MP treatment (10 mg/kg every 12 hr for 9 doses) beginning on Day 2 prevented the increase in MI and LI seen at Day 4 after CP and ADR alone. The results indicate that recovery was delayed until after cessation of MP treatments, at which point increases in LI and MI were noted between 12 and 24 hr after the last dose of MP.
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Table 1 shows the response of C3H/HeJ mammary tumors to MTX and FUra given at various times after CP and ADR with or without MP. The tumor responses are expressed in terms of regrowth delay and time to double pretreatment size. All treatment groups contained at least 5 tumor-bearing mice.

The results show that MTX and FUra alone or MP every 12 hr for 9 doses had little effect on tumor growth. The addition of ADR 24 hr after CP did, however, increase the regrowth delay from that seen with CP alone. MTX and FUra given on Days 2 or 6 after CP and ADR were no better than CP and ADR alone. When MTX and FUra were given on Day 4, significant bone marrow toxicity resulted. As might be predicted, however, animals surviving the bone marrow toxicity demonstrated relatively good tumor responses. The mean time to reach pretreatment size and mean time to reach twice pretreatment size for this group does not include values for 2 of 6 surviving mice which, although never attaining a complete remission, died with time to reach pretreatment size in excess of 90 days.

MP treatment beginning on Day 2 after CP did not prevent bone marrow toxicity as seen by the 60% 30-day death in this group. Tumor responses were generally poor in these mice, but the severe toxicity precluded adequate measurement and evaluation. MTX and FUra on Day 6, 2 hr after the last MP dose, while being slightly less toxic, was no more effective than were CP and ADR alone. When FUra and MTX were, however, administered at 6.5 days, a time following cessation of MP when the [3H]dThd LI was increasing, better regrowth delay was observed than with CP and ADR alone or with CP, ADR, MP, and MTX plus FUra given at a kinetically inappropriate time.

DISCUSSION

Changes in the cell kinetics following chemotherapeutic drugs and radiation have been useful for predicting efficacious time-sequenceing intervals in a variety of experimental animal tumors (4, 6–9, 15, 17). Glucocorticosteroids have been shown by many investigators to inhibit cell proliferation in many in vitro systems (12, 15, 18). In these systems, the data are consistent with a G1 progression delay. In vivo hydrocorticos-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TVo (days)</th>
<th>TDV (days)</th>
<th>30-day toxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>10.1 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>CP (200 mg/kg)</td>
<td>6.1 ± 0.2</td>
<td>16.3 ± 1.8</td>
<td>0</td>
</tr>
<tr>
<td>FUra (42 mg/kg) + MTX (6 mg/kg)</td>
<td>4.7 ± 0.5</td>
<td>14.6 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td>MP every 12 hr for 9 doses (10 mg/kg)</td>
<td>4.5</td>
<td>14.4 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>CP + ADR (2 mg/kg) on Day 1</td>
<td>13.8 ± 1.9</td>
<td>20.3 ± 2.1</td>
<td>0</td>
</tr>
<tr>
<td>CP + ADR + (FUra + MTX, Day 2)</td>
<td>13.9 ± 0.7</td>
<td>21.1 ± 1.6</td>
<td>0</td>
</tr>
<tr>
<td>CP + ADR + (FUra + MTX, Day 4)</td>
<td>20.5 ± 2.6</td>
<td>28.3 ± 4.8</td>
<td>45</td>
</tr>
<tr>
<td>CP + ADR + (FUra + MTX, Day 6)</td>
<td>13.1 ± 2.5</td>
<td>20.6 ± 1.9</td>
<td>40</td>
</tr>
<tr>
<td>CP + ADR + MP + (FUra + MTX, Day 4)</td>
<td>17.0 ± 0.8</td>
<td>21.5 ± 2.5</td>
<td>25</td>
</tr>
<tr>
<td>CP + ADR + MP + (FUra + MTX, Day 6)</td>
<td>21.4 ± 1.2</td>
<td>32.1 ± 3.1</td>
<td></td>
</tr>
</tbody>
</table>

* TVo, time to reach pretreatment size; TDVo, time to reach twice pretreatment size.
* Mean ± S.E.
* Includes only regrowing tumors; 2 of 6 mice died prior to reattaining V0 with TVo in excess of 90 days (see text).
* 10 mg/kg every 12 hr for 9 doses beginning on Day 2.
teroids have been shown to induce G1 progression delay in leukemia (14, 17) and in C3H/HeJ spontaneous mammary tumors (9). The results from the present studies indicate that proliferative recovery in C3H/HeJ mammary tumors after CP and ADR can be delayed by instituting MP treatments at a time prior to the expected recruitment of cells destined to repopulate the tumor. In the context of the design of sequential chemotherapy protocols with CP, ADR, FUra, and MTX, cell kinetic considerations alone, while resulting in good tumor control, resulted in unacceptable marrow toxicity.

MP treatment beginning on Day 2 after CP and ADR, while not affecting the onset of bone marrow recovery, did reduce the compensatory hyperproliferation noted between Days 3 and 4. Toxicity resulting from Day 4 treatment with MTX and FUra was not prevented with MP. MP treatment did, however, delay or prevent the recruitment of cells destined to repopulate the tumor and afforded the bone marrow sufficient recovery time such that the toxicity resulting from the cell kinetically based sequential chemotherapy with MTX and FUra was reduced to an acceptable level.

As has been noted in other studies (4, 6, 8, 9, 23), cell kinetic parameters derived by in vitro methods after perturbation can predict efficacious and non efficacious treatment times. This was best demonstrated by the differences between the response when MTX and FUra were given at the time of the last MP treatment, when the [3H]dThd LI was low, and the superior response when the MTX and FUra were given at the time when the [3H]dThd LI was increasing after cessation of MP treatments.

Synergistic responses have been observed when MTX is administered prior to (1, 11, 13, 16) or simultaneously with (13) FUra. In L1210 leukemia, the schedule-dependent synergism is apparently the result of MTX-mediated increases in intracellular phosphoribosylpyrophosphate which may promote increased intracellular accumulation of FUra (10). It is conceivable that the short interval (less than 15 min) between MTX and FUra in the present studies was suboptimal. If so, a better response might be predicted with the appropriate interval between MTX and FUra treatments.

From a clinical viewpoint, the results from this and other studies (9) indicate that, in corticosteroid-sensitive tumors, concurrent treatment with steroids and chemotherapeutic agents may be contraindicated and that the effectiveness of cycle stage-specific agents may be considerably reduced due to unfavorable cell cycle distributions. On the other hand, corticosteroids when used appropriately may add significantly to the effectiveness of a sequential chemotherapy program by altering the temporal relationship of the recovery events in the tumor and the normal tissue.

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