Effect of Methylprednisolone on Cell Proliferation in C3H/HeJ Spontaneous Mammary Tumors

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ABSTRACT

The present studies were initiated to investigate the effects of methylprednisolone (MP) on the growth and cell kinetics of C3H/HeJ spontaneous mammary tumor. MP given every 12 hr in 9 doses at 10 or 20 mg/kg/dose resulted in temporary stasis of tumor growth with little or no volume regression. Cell kinetic parameters were determined by in vitro methods after MP every 12 hr in 3 doses. At both dose levels a similar 50% decrease in the tritiated thymidine labeling index was observed 2 hr after treatment. At this time, the primer-dependent DNA polymerase labeling index, an in vitro estimate of tumor growth fraction, was similar to untreated controls at both dose levels. The cell kinetic changes after MP (10 mg/kg every 12 hr in 3 doses) suggested a reversible G1 block with synchronous progression of tumor cells through the cell cycle after cessation of treatment. While synchrony was also suggested after MP (20 mg/kg every 12 hr in 3 doses), resumption of cell proliferation was delayed by 18 hr. Treatment with 5-fluorouracil and methotrexate 12 hr after cessation of MP (10 mg/kg every 12 hr in 3 doses), a time corresponding to maximal tritiated thymidine labeling index, resulted in greater tumor regression and greater regrowth delay than did 5-fluorouracil and methotrexate given at times corresponding to low tritiated thymidine labeling indexes. Tumor volume-doubling times during regrowth were significantly longer than either prior to treatment or after methotrexate and 5-fluorouracil alone.

INTRODUCTION

GCS2-specific receptors have been identified and quantitated in a variety of experimental tumor systems (3, 9, 13, 16, 18, 23, 26, 28), their corresponding normal tissue (2, 15), human leukemia (22, 23, 29, 35), and breast cancer (12). Although GCS2 has been shown to stimulate cell division (36), the growth of many cultured cell lines (10, 17–19, 27, 29–31), proliferation of ileal epithelium and spleen cells (20), and numerous membrane functions (14, 38) can be inhibited by GCS. Stimulation of DNA synthesis in lymphocytes by phytohemmagglutinin (26, 28) and in 3T3 cells by prostaglandins (1) could be inhibited by GCS. Further, the degree of DNA synthesis inhibition in cultured breast cancer cells was directly proportional to GCS receptor content (26). Studies with acute leukemia cells (11, 24, 25), HeLa cells (21), and rat pituitary tumor cells (10) have shown that GCS can induce a block in cell cycle progression near the G1-S boundary.

Although the effects of GCS on tissue culture and leukemia cells have been documented, much of the information regarding the effects of these agents on cell proliferation has not been integrated into clinical practice. Although GCS2 are widely used together with other agents in solid tumor therapy, little or no information is available on the effects of GCS on the proliferation kinetics of solid tumors. The present studies were initiated to investigate the effects of MP on the growth and cell kinetics of spontaneously arising mammary tumors in C3H/HeJ mice.

MATERIALS AND METHODS

Animals. Retired breeder female C3H/HeJ mice were maintained in our animal facility and checked weekly for the appearance of tumors. Animals were maintained on a 12-hr light-dark cycle and fed Purina laboratory chow (Ralston Purina, Evansville, Ind.) and water ad libitum. Tumor-bearing animals were culled weekly, and tumors were measured by callipers in 3 dimensions. Tumor volumes were estimated from volume = (length × width × height)/2. Tumor measurements were made at least weekly prior to treatment and at closer intervals after treatment. Tumor volume-doubling times were calculated as previously described (5).

Drug Treatments. Tumor-bearing mice were weighed and given MP (Solumedrol; The Upjohn Co., Kalamazoo, Mich.) i.p., when tumor volumes were approximately 0.5 to 0.8 cu cm. MP was administered at either 10 or 20 mg/kg every 12 hr for either 3 or 9 injections. Animals were killed at various intervals after cessation of MP treatments, and cell kinetic measurements were made. In some studies MTX (6 mg/kg) and 5-FU (42 mg/kg) were administered i.p. in 0.9% NaCl solution. In some studies hematoxylin and eosin-stained paraffin sections were prepared for histological examination.

Cell Kinetic Measurements. The methods for in vitro single and double labeling of solid tumors have been described in detail previously (4, 5). Briefly, tumor-bearing animals were killed by cervical dislocation. The tumors were resected and minced in McCoy's media supplemented with fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The tumor cell suspensions were then either singly labeled with [3H]dThd (15 to 17 Ci/mmol, 2.5 μCi/ml; Amersham, Arlington Heights, Ill.) for 30 min or double-labeled with [3H]dThd for 1 hr and then with [14C]dThd (54...
Effect of MP on Cell Proliferation in C3H/HeJ SMT

little or no tumor regression was noted during treatment, no significant increase in tumor size was observed at either the 10- or 20-mg/kg dose levels until after cessation of MP treatments. Posttreatment growth rates were similar to the growth rate prior to treatment.

Chart 2 shows the cell kinetic response to MP (10 mg/kg every 12 hr in 3 doses). \([^{3}H]dThd\) LI's were reduced 2 hr after the last MP dose by approximately 50%. PDPI and DLI were not significantly different from control. If DLI is assumed to be equivalent to \(T_{51}\), cell cycle times and cell production rates computed by the formulations of Steel (34) were approximately 55 hr and 0.00304/hr, respectively, 2 hr after MP. Within 12 hr after the last MP dose the \([^{3}H]dThd\) LI was increased to approximately 14.0%. The PDPI was unchanged and was not significantly different from the \([^{3}H]dThd\) LI. The PDPI increased between 18 and 42 hr after

\[ \frac{(T_{51})(^{3}C-labeled\ cells)}{(^{3}H\ only\ labeled\ cells)} \]

where \(T_{51}\) is the interval between administration of the 2 labeled dThd's (4, 38). It should be recognized that this expression reflects the entry of S phase cells into \(G_{2}\) and that only during asynchronous cell cycle progression does it truly reflect the \(T_{51}\). During synchronous proliferation the calculated \(T_{51}\) could be artificially prolonged if the synchronized cohort is in early S phase (i.e. few cells making the S-G2 transition) or artificially short if the synchronized cohort is at the S-G2 transition at the time of assay. Therefore, we will refer to the calculated \(T_{51}\) values as a DLI. Cell cycle times and cell production rates were estimated as previously described (5) with the formulations of Steel (34). Student's \(t\) test was used for statistical analysis.

RESULTS

Chart 1 shows the growth for C3H/HeJ SMT treated with MP (10 or 20 mg/kg MP every 12 hr in 9 doses). Although
treatment and a secondary increase in \([\text{H}^3\text{H}]\text{dThd LI}\) was noted at 42 hr after treatment. DLI's were profoundly increased at 12 and 36 hr after MP; however, the DLI was markedly shorter than at normal at 18 and 42 hr.

The cell kinetic response after MP (20 mg/kg every 12 hr in 3 doses) is shown in Chart 3. As in the 10-mg/kg study, the \([\text{H}^3\text{H}]\text{dThd LI}\) was reduced by approximately 50%, 2 hr after the last MP dose. Although histological sections revealed reduced mitotic activity at this time, little or no overt cell destruction or necrosis was noted at either dose level. PDPI's were similar to untreated control for the first 24 hr after treatment, while \([\text{H}^3\text{H}]\text{dThd LI}\)'s were subnormal until 24 hr after MP. Recovery of the \([\text{H}^3\text{H}]\text{dThd LI}\) was initiated between 18 and 24 hr with maximal labeling at 30 hr. Increases in the PDPI were not noted until 30 hr, with maximal PDPI (50%) seen at 42 hr after MP. At 60 hr the PDPI was similar to control. The DLI was increased between 2 and 30 hr after the last MP treatment, while subnormal DLI's were noted at 36 and 60 hr after treatment.

Inasmuch as MP (10 mg/kg every 12 hr in 3 doses) was apparently quite effective in partially synchronizing the tumor cell population, studies to determine whether this synchronizing effect could be exploited for increased therapeutic benefit were initiated. Chart 4 shows the results from studies in which animals were treated with simultaneous MTX (6 mg/kg) plus 5-FU (42 mg/kg) alone or at 2, 12, or 24 hr after MP (10 mg/kg every 12 hr in 3 doses). The results show that, while only a modest tumor regression was seen after MTX plus 5-FU alone (18%), approximately 58% tumor volume regression was noted in replicate experiments when MTX plus 5-FU were given at the time, 12 hr, when most of the proliferating cells were synchronized in S phase. When 5-FU plus MTX was administered at times corresponding to low \([\text{H}^3\text{H}]\text{dThd LI}\) (2, 24 hr), little or no effect on tumor size was noted. While necrosis in untreated size-matched tumors is uncommon (5), extensive cell destruction and necrosis were observed in histological sections within 48 hr when MTX and 5-FU were given 12 hr after MP. In the MTX plus 5-FU group, tumors regained pretreatment size by Day 5 while in the MP plus 5-FU plus MTX (12 hr) group pretreatment sizes were not obtained until Day 20. The tumor volume-doubling times calculated during the first 18 days of tumor regrowth from the MP plus 5-FU plus MTX group [19.5 ± 1.5 (S.E.) days] were significantly longer than pretreatment doubling times (12.5 ± 1.0 days) or doubling times for regrowing tumors treated with MTX plus 5-FU alone (9.7 ± 1.1 days).

DISCUSSION

In the present studies MP at 10 and 20 mg/kg every 12 hr in 9 doses retarded C3H/HeJ SMT growth. Studies with 7,12-dimethylbenz(α)anthracene-induced rat mammary tumors showed enhanced tumor growth after adrenalectomy and growth suppression after high daily doses of hydrocortisone acetate (8). Although GCS-specific protein receptors have been identified in 7,12-dimethylbenz(α)anthracene-induced rat mammary tumors (15), the stimulation of growth after adrenalectomy may have been related in increased levels of prolactin (8). While indirect effects of MP in the present experiments cannot be ruled out, cell kinetic measurements after 10 and 20 mg/kg (every 12 hr in 3 doses) suggested suppression of tumor cell proliferation with synchronous progression through the cell cycle after cessation of treatment. GCS's have been previously shown to affect a reversible G1 block in cell cycle progression in acute leukemia (25), in phytohemagglutinin-stimulated lymphocytes (28), and in HeLa cells (21).

At 2 hr after MP every 12 hr in 3 doses, the DLI and the growth fraction, as estimated by the PDPI, were similar to untreated controls. Decreases in the \([\text{H}^3\text{H}]\text{dThd LI}\) suggested reduced S-phase cellularity. If the normal DLI is assumed to be \(T_s\), calculated cell cycle times were approximately dou-
bled (55 hr) as compared to values (30 hr) for untreated spontaneous mammary tumors (5). Previous studies with GCS-treated HeLa cells have also shown increased cell cycle times without concomitant changes in $T_s$ or growth fraction (21). Continuous labeling and flow microfluorimetry studies with rat pituitary tumor cells have also shown that, while not effecting the growth fraction, GCS treatment did result in decreased S-phase cellularity with concomitant $G_2$ accumulation (10).

The observed tumor growth rate can be defined as the difference between the rate of cell production and the rate of cell loss (34). If cell production is balanced by cell loss, then no growth is observed. Two hr after MP (10 mg/kg every 12 hr in 3 doses), the cell production rate, calculated by the method of Steel (34), was approximately 0.00304/hr. This is very similar to the previously reported rate of cell loss (0.00344/hr) for C3H/HeJ SMT (5). Thus, if MP treatment did not radically alter the cell loss rate, the observed effect of MP on tumor growth is consistent with the level of decreased cellular proliferation.

Although the fraction of S-phase cells ([3H]dThd LI) is a component of the growth fraction (measured by the PDP), the 2 parameters are not interchangeable. While all S-phase cells are PDPI-positive, it is clear that non-S-phase cells also contribute to the PDPI (32). Therefore, changes in one or both parameters may imply different kinetic behavior patterns. For example, increases in both parameters may imply an increase in the size of the proliferating pool, while increases in the [3H]dThd LI without a change in PDPI may imply increased accumulation of cells in S phase without an expansion of the proliferating pool.

There is substantial evidence from the present experiments with 10-mg/kg schedules that following cessation of treatment C3H/HeJ mammary tumor cells progress synchronously through the cell cycle. At 2 hr after treatment, the [3H]dThd LI is decreased 50% with little or no effect on PDPI or DLI. This would suggest that a portion of the cell population is blocked in $G_1$. Increases in [3H]dThd LI at 12 and 42 hr after cessation of treatment would suggest synchronous cell cycle progression. [3H]dThd LI's at 12 hr were similar in magnitude to the PDP, suggesting that most of the proliferating cells were in S phase at this time. Increased PDPI following the increase in [3H]dThd LI may be reflective of newly formed daughter cells remaining in cycle. In contrast, the decreased proliferation after MP resulted from nonspecific blockage or random slowdown of cycle progression, one might have expected a recovery of kinetic values to normal levels with little or no overshoot and no secondary increase in [3H]dThd LI. Following release from the MP-induced block, cell cycle progression was apparently normal, since the 30-hr interval between [3H]dThd LI maxima is compatible with previously established cell cycle times for unperturbed C3H/HeJ SMT (5, 7).

In our studies the [14C,3H]dThd double label method was used to monitor the movement of cells through DNA synthesis. During asynchronous conditions this method can be used to estimate $T_s$, while during synchronous cell cycle progression it only reflects the movement of S-phase cells into $G_2$.

While it is not possible to evaluate quantitatively the extent of synchronization or the age distribution of the synchronized cohort, the changes in the DLI are qualitatively compatible with the $G_2$ entry expected based on the [3H]dThd labeling data. For example, increased DLI values (subnormal $G_2$ entry) were noted at times when the S-phase population was increasing (12 and 36 hr), while subnormal DLI values (increased $G_2$ entry) were noted when the S-phase population was decreasing (18 and 42 hr). Since the DLI would be rapidly changing as the cohort moved through S phase and into $G_2$, considerably shorter study intervals would be necessary to evaluate quantitatively the age distribution and S-phase transit of the synchronized cohort.

The response after MP (20 mg/kg every 12 hr in 3 doses) was similar to the response at the 10-mg/kg dose level in that [3H]dThd LI's were reduced to a similar extent and that the recovery pattern, characterized by increased [3H]dThd LI's and abruptly changing $G_2$ entry, suggested synchronization. In contrast, however, recovery was delayed approximately 18 hr as compared to the 10-mg/kg study. The finding of substantially increased DLI and its implied reduced $G_2$ entry at 2 hr in the 20-mg/kg study is difficult to explain. Although this observation at the high MP dose level could be a result of nonspecific membrane effects on dThd uptake (17, 37), it could also suggest that, in addition to $G_2$ blockage, other points in the cell cycle (possibly S phase) may be sensitive at this dose level.

While chronic MP treatment (every 12 hr in 9 doses) at either dose level did not result in any significant decrease in tumor volume or any obvious increased cell destruction, treatment with MTX and 5-FU 12 hr after MP (10 mg/kg every 12 hr in 3 doses), a time of maximal S-phase cellularity, resulted in approximately 60% tumor regression, slow tumor regrowth, and histological evidence of extensive tumor cell destruction by 48 hr after treatment. In contrast, 5-FU plus MTX at either 2 or 24 hr after MP resulted in little or no effect on tumor regression. These observations lend further support to the proposed synchronizing action of MP, since the low [3H]dThd LI's at these times would suggest a relatively small fraction of potentially sensitive cells.

Although GCS-induced cell kinetic perturbations have been observed in a variety of experimental tumor systems, the therapeutic potentials of these alterations have not been tested in the clinic. In fact, high doses of GCS are frequently used simultaneously with cell cycle stage-specific agents in solid tumor chemotherapy. The cell kinetic and treatment results from our studies together with the results from many other studies might indicate that, in solid tumors sensitive to GCS, the effectiveness of cycle active agents could be reduced when administered simultaneously with GCS. Whether synchronization with one or more steroid hormones and subsequent kinetically based cell cycle stage-specific chemotherapy would be more effective for tumor control and survival than conventional chronic hormonal therapy will require further studies with human as well as experimental solid tumors.

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