Enhanced Radiation Lethality in Partially Synchronized Solid Mouse Tumors

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ABSTRACT

We studied the combined effects of local irradiation on in vivo partially synchronized solid mouse tumors. Syngeneic fibrosarcoma cells were transplanted s.c. into the thighs of C3H/He mice. When the tumors grew to 179 cu mm in volume, 1-β-D-arabinofuranosylcytosine (ara-C) was repeatedly injected i.p. followed by a single injection of vinblastine sulfate at 5 hr after the end of the ara-C treatment. The mitotic indexes increased from 4% in control to 22 to 23% at 5 hr after the ara-C treatment, and the level continued for another 5 hr. Further treatment with vinblastine sulfate after the ara-C injections resulted in more effective accumulation of mitotic cells, i.e., 30% at the sixth hr. The tumor was locally irradiated with a single dose of 3000 rads of γ-rays at the maximum level of mitotic index. The results indicated a synergistic inhibition of tumor growth and an 84% prolongation of the 50% survival day beyond that of the nontreated control mice.

INTRODUCTION

Radiotherapy might be more effective if solid tumor cells were synchronized and accumulated in a radiation-sensitive phase using cell cycle-dependent drug(s). Since the first report of Terasima and Tolmach (13), the mitotic phase has been widely accepted as the most radiosensitive phase in the cell cycle (3).

In vitro partial synchronization of mammalian cells by administration of ara-C under appropriate conditions was reported by Kim and Eidinoff (7). They treated HeLa S-3 cells with ara-C (8.2 × 10^-6 M) for one generation and observed increased S-cell fraction and partially synchronized cell growth shortly after removal of ara-C. Application of this method to in vivo tumor cells was performed by Gibson and Bertalanffy (4). They have reported that repeated i.p. injections of ara-C into the mice bearing B16 melanoma resulted in a partial synchronization of the tumor cells. Although there was no description of labeling and mitotic indexes shortly after ara-C treatment, their data suggested to us that a drastic increase of mitotic cell fraction might be found before the increase of labeling indexes. The duration of the mitotic phase, however, is usually less than 1 hr, and it is difficult to find the most effective timing for irradiation. To avoid such instability and to accumulate many more cells in the mitotic phase, we have used VBL as the most suitable drug (12).

In our experiments, successful accumulation of mitotic cells was produced by a further treatment with VBL after the multiple injection of ara-C in the NR-FS tumor. The purpose of this study was to investigate the combined effects of drugs and local irradiation on in vivo partially synchronized solid mouse tumors and on the survival time of the tumor-bearing mice.

MATERIALS AND METHODS

Animal Tumor System. Equal numbers of male and female C3H/He mice (8 to 12 weeks old) bred in specific-pathogen-free animal facilities were used in these experiments. The fibrosarcoma, designated NR-FS, originated from a spontaneously developed tumor in a male C3H/He mouse and was maintained for more than 36 generations in our laboratory.

Tumor Cell Transplantation. Isotransplants at the 35th generation were removed and minced with scissors so as to make each piece less than 1 cu mm in volume. The mince was poured into a beaker with 75-mI Dulbecco's calcium-and-magnesium-free PBS containing 0.05% trypsin (1:250; Difco Laboratories, Inc., Detroit, Mich.) and stirred gently with a magnetic stirrer at 37°. The initial yield after agitation for 10 min was discarded to remove debris and dead cells. The supernatant was centrifuged for 5 min at 1800 rpm, and the sediment was diluted with Hanks' balanced salt solution containing 5% fetal calf serum. When the tumor cell suspension (1.05 × 10^6/10 μl viable tumor cells) was transplanted s.c. into the right thigh, tumor volume reached 179.0 ± 37.0 (S.D.) cu mm at Day 7. These tumors were then subjected to synchronization and irradiation.

Autoradiography. The isotope used to label S-phase cells was [3H]thymidine, with a specific activity of 5.0 Ci/mmol (The Radiochemical Centre, Amersham, England). When tumor volume had reached 179 cu mm, each mouse was given an i.p. injection of 50 μCi/mouse in 0.1 ml sterile PBS. Mice were killed at various intervals from 1 to 30 hr after a single administration of [3H]thymidine. Tumor tissues were removed, fixed with 10% PBS-formalin, embedded in paraffin, and sectioned 4 μm thick. Four sections from a tumor sample were dipped into Sakura NR-M2 nuclear emulsion (Konishiroku, Tokyo, Japan), stored in a refrigerator for 21 days at 4°, developed, and stained with hematoxylin-eosin solution. The cells which had more than 3 grains were defined as labeled. Background count was less than 1 grain/cell.

More than 200 mitotic cells in each sample were randomly investigated to obtain the FLM curve. Cell cycle parameters from the FLM curve were calculated by the method of Takahashi et al. (11).

Mitotic Index. Four sections from a sample were stained with hematoxylin-eosin solution. The mitotic index was determined by the number of cells undergoing mitosis per 1000 cells and was expressed as a percentage.

Local Irradiation. Tumors at a mean volume of 179 cu mm were irradiated. Mice were anesthetized with sodium Nembutal (0.07 mg/g body weight) (Abbott Laboratories, Chicago, Ill.) and taped to a brass plate. The tumor-bearing leg was then stretched and centered over a circular aperture (3 cm in diameter) of the plate. The mouse, fixed on the plate, was placed into the irradiator and moved over at one-half of the programmed dose in order to irradiate the tumor region uniformly.

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The dose rate, measured with a LiF thermoluminescent dosimeter, was 350 rads/min at 15 cm from the $^{137}$Cs source to the center of the tumor. A single dose of 3000 rads $\gamma$-rays was given to each mouse tumor.

**Tumor Growth Curve, TG Time, and TDG Time.** A tumor was regarded as an ellipsoid with 3 diameters: a, b, and c. Each diameter was measured by a caliper at 2- or 3-day intervals. The formula, $a b c/6$, was used to calculate tumor volume. The tumor growth curves were obtained by plotting the volume on a semilogarithmic section paper. The TG time was estimated by the method described by Ando et al. (1), i.e., the time required for a tumor to reach 1000 cu mm after treatment was obtained on the section paper. A difference between the TG times of a treated and a nontreated tumor was defined as TDG time (1).

$SD_{50}$. SD$_{50}$ was calculated by the logit analysis method on the basis of the number of days which the mice survived after treatment.

**Statistical Analysis.** All raw data were pooled and evaluated statistically by Student's t test or Welch-Aspin test, depending on the scope of the variances (determined by $F$ test). A difference between groups was considered significant if $p$ was 0.05 or less.

**Drugs.** ara-C (Nippon Shinyaku Co., Ltd., Kyoto, Japan) was dissolved in PBS at a concentration of 0.2 mg/ml. VBL (Shionogi Co. Ltd., Osaka, Japan) was dissolved in PBS at a concentration of 0.2 mg/ml. These solutions were injected i.p. into tumor-bearing mice.

**RESULTS**

**Cell Cycle Time.** Thirty-six tumors in the exponential growth phase were subjected to investigation for the FLM. Both the FLM countings and the computer-fitted curve are shown in Chart 1. The computer analysis revealed that the first peak in the FLM curve reached its maximum of 99% at 4 hr after $[^3]H$thymidine injection, and the first trough appeared at 16 hr. The mean cell cycle time ($T_c$) and mean time durations for $G_1$, $S$, $G_2$, and $M$ phases were determined and are listed in Table 1.

**In Vivo Synchronization.** The protocols for inducing in vivo synchrony of NR-FS tumor by ara-C and VBL. ara-C injection had started when average tumor volume had reached 179 cu mm after the last injection of ara-C. They were given injections of VBL (0.5 mg/kg body weight), and the mitotic frequency was examined as before.

Changes in mitotic index after the last injection of ara-C are shown in Chart 3. The mitotic indexes increased from about 4% in the nontreated control group to approximately 22 to 23% at 5 hr after the ara-C treatment, and the level continued for another 5 hr. The second peak of the mitotic indexes reached about 25%, a fact which might be attributed to the cells accumulated at the region of $G_1\rightarrow S$ transition during ara-C treatment. A further increase in mitotic frequency by additional treatment with VBL reached 29% (Chart 3). This increase was explained by the accumulation of mitotic cells due to the stathmokinetic effect of VBL.

In the microscopic examination, only a few mitotic cells could be seen in the section of nontreated tumor. By contrast, an appreciable increase of mitotic cells was observed in the section of the ara-C-treated tumor (Fig. 1).

**Effects of Local Irradiation on In Vivo Synchronized Tumor.** Local irradiation was performed in accordance with the schedule illustrated in Chart 4. After a single dose of 3000 rads of $\gamma$-ray irradiation, the tumor volume in each series of mice was measured periodically until the 20th day. According to the growth curve obtained from several series of experiments (Chart 5), the TG time for each modality of treatment was calculated and is shown in Table 2. Neither a single injection of ara-C (Modality B) nor sole administration of VBL (Modality C) showed any significant inhibitory effect on the tumor growth. The tumor growth of the mice given injections of ara-C 8 times at 2-hr intervals (Modality D) was suppressed slightly for 15 days in the growth curve and TG time but reached control level thereafter. $\gamma$-Irradiation (Modality E), however, produced an appreciable delay of tumor growth (Chart 5), and the TDG time of this series was 4.7 days (Table 2). When the tumor was irradiated after repeated injections of ara-C (Modality F), the TDG time increased to 10.3 days. Value F, which may be
thought of as the observed value, was statistically not significant in comparison with the sum of the Values D and E as the expected value, indicating that the repeated ara-C injection additively increased radiation lethality. In the last series of experiments, irradiation was performed after repeated ara-C treatment and an additional single injection of VBL (Modality G). Using this modality (G), the tumor growth was the most intensively suppressed (Chart 5), and the TGD time was prolonged to 15.0 days (Table 2). The difference between the observed and the expected value was significant in Treatment G. Thus, it would appear that combining the respective treatments based on cell population kinetics, as in Modality G, is more effective than giving them independently. The repeated treatments of ara-C plus VBL in combination with γ-irradiation resulted in more inhibitory effects than expected; i.e., the effects were synergistic rather than additive. These enhanced radiation effects might have resulted from an increased fraction of sensitive cells, namely, mitotic cells.

![Chart 3](image)

**Chart 3.** Changes in mitotic indexes of NR-FS tumors after the last injection of ara-C. •, changes after multiple ara-C injection; ○, mitotic index in nontreated control; ○, changes after combination treatment of ara-C and VBL.

![Chart 4](image)

**Chart 4.** Schedules of combined radiotherapy with in vivo partial synchronization for NR-FS tumors and other control treatments.

![Chart 5](image)

**Chart 5.** Tumor growth curves after combined treatment of local γ-ray irradiation, in vivo partial synchronization, and other control treatments.

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Fig. 1. Photomicrograph showing an appreciable increase of mitotic cells in the section of the NR-FS tumor extirpated at 7 hr after the last injection of ara-C. ara-C (10 mg/kg) was administered i.p. 8 times at 2-hr intervals. H & E. x 1000.
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Table 2
TGD time after treatment

<table>
<thead>
<tr>
<th>Modality of treatment</th>
<th>TGD time (days)</th>
<th>( \rho (t \text{ test})^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Nontreated</td>
<td>4.63 ± 0.45b</td>
<td>0.09  ± 0.05</td>
</tr>
<tr>
<td>B. ara-C^c</td>
<td>0.39 ± 1.30</td>
<td>0.39 ± 1.30</td>
</tr>
<tr>
<td>C. VBL^d</td>
<td>1.57 ± 0.10</td>
<td>0.47 ± 0.27</td>
</tr>
<tr>
<td>D. ara-C + VBL + γ-ray^g</td>
<td>10.27 ± 3.78</td>
<td>15.04 ± 2.12</td>
</tr>
<tr>
<td>E. γ-Ray^j</td>
<td>6.73 ± 2.47</td>
<td>0.39 ± 0.27</td>
</tr>
<tr>
<td>F. ara-C^c + γ-ray^g</td>
<td>7.27 ± 3.37</td>
<td>116.9 ± 33.6</td>
</tr>
<tr>
<td>G. ara-C^c + VBL + γ-ray^g</td>
<td>116.9 ± 33.6</td>
<td>116.9 ± 33.6</td>
</tr>
</tbody>
</table>

\( ^a \) To estimate statistical significance, the following equation was used.

For Modality F,

\[ f = \frac{F - (D + E)}{\sqrt{SD_c^2 + SD_D^2 + SD_E^2}}. \]

For Modality G,

\[ f = \frac{G - (C + D + E)}{\sqrt{SD_c^2 + SD_D^2 + SD_E^2}}. \]

where \( C, D, E, F, G, SD_c, SD_D, SD_E, SD_G \) are mean TGD time and their SD for each modality of treatment, respectively.

\( ^b \) Single injection of ara-C (10 mg/kg body weight).

\( ^c \) NS, not significant.

\( ^d \) Single injection of VBL (0.5 mg/kg body weight).

\( ^e \) Eight injections of ara-C (10 mg/kg) at 2-h intervals.

\( ^f \) Single dose of 3000 rads γ-ray irradiation on the tumor site.

Table 3
SDso of tumor-bearing mice treated when mean tumor volume reached 179 cu mm.

<table>
<thead>
<tr>
<th>Modality of treatment</th>
<th>SDso (days)</th>
<th>Rate of prolongation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Nontreated</td>
<td>18.3 (16.4-20.4)</td>
<td>100.0</td>
</tr>
<tr>
<td>B. ara-C^c</td>
<td>18.9 (17.7-20.1)</td>
<td>103.3</td>
</tr>
<tr>
<td>C. VBL^d</td>
<td>18.6 (17.1-20.2)</td>
<td>101.6</td>
</tr>
<tr>
<td>D. ara-C^c + γ-ray^g</td>
<td>15.5 (13.8-17.4)</td>
<td>84.7</td>
</tr>
<tr>
<td>F. ara-C^c + VBL + γ-ray^g</td>
<td>24.4 (22.6-26.1)</td>
<td>133.3</td>
</tr>
<tr>
<td>G. ara-C^c + VBL + γ-ray^g</td>
<td>33.6 (32.2-35.0)</td>
<td>183.6</td>
</tr>
</tbody>
</table>

\( ^g \) Numbers in parentheses, 95% confidence limits.

\( ^h \) Single injection of ara-C (10 mg/kg body weight).

\( ^i \) Single injection of VBL (0.5 mg/kg body weight).

\( ^j \) Eight injections of ara-C (10 mg/kg) at 2-h intervals.

\( ^k \) Single dose of 3000 rads γ-ray irradiation on the tumor site.

**Effects of Combined Therapy with Local Irradiation and in Vivo Synchronization of Survivals.** SDso of tumor-bearing mice either nontreated or treated with drugs and/or γ-rays is shown in Table 3. The most remarkable prolongation of SDso, 84% beyond that of nontreated control mice, was found in those mice which were locally irradiated with γ-rays in combination with in vivo synchronization by ara-C and VBL.

Both the combination of γ-ray irradiation with 8 injections of ara-C and a single irradiation of 3000 rads succeeded in prolonging the SDso by 33 and 17%, respectively, in comparison with the nontreated control group. Neither single injection of ara-C nor sole administration of VBL caused any significant prolongation. The SDso of the mice treated with 8 ara-C injections alone was shorter than that of the nontreated control group.

**DISCUSSION**

In this study, we investigated the effects of combining radiation therapy with in vivo synchronization by using a suitable regimen of ara-C and VBL administration for the originally asynchronous cell population of NR-FS solid tumors. Terasima and Tolmach (13) and Sinclair and Morton (10) indicated with cultured mammalian cells that the radiosensitivity of tumor cells depends upon their cell cycle age and that both the M phase and G1-S transition are the most sensitive to radiation. It may also be expected in the solid tumor that the greater the number of cells in the radiosensitive phase of the cell cycle, the larger the radiation lethality.

Gibson and Bertalanffy (4) obtained a sizable degree of cell cycle synchrony in a solid B16 melanoma by repeated injection of ara-C. They proved that the synchronized cohort of cells entering simultaneously into the S phase yielded a high percentage of cells incorporating [3H]thymidine. Immediately after the synchronized cohort of cells passed through the S phase into the G2 phase, the percentage of cells incorporating [3H]thymidine declined.

It is well known that the majority of cells in G2, M, or G1, at ara-C administration proceed normally through the cell cycle to the terminus of G1, or to the G1-S transition, where they are blocked (6). Those cells in the S phase are either arrested immediately by a single injection of ara-C or inactivated. The degree of cell inactivation in the S phase depended on the dosage of ara-C (6). Because a toxic effect on the tumor-bearing mice was observed in our preliminary experiments using the dose of 12.5 mg/kg reported by Gibson and Bertalanffy (4), a slightly lower dosage (10 mg/kg) was selected.

Since the effects of ara-C at an optimum concentration on cell progression from G2 to the terminus of the G1 phase would be small (6, 7), the minimum period of ara-C treatment depends on the mean duration of the G2, M, and G1 phases. The total duration of the G2, M, and G1 phases of exponentially growing NR-FS tumor cells was approximately 11 hr. This means that a minimum of 11 hr might be required to accumulate a majority of the growing cells into the S phase. Considering the variation in cell progression rates, and referring to the best case reported by Gibson and Bertalanffy (4), we have treated tumor-bearing mice for 14 hr, a period which is equivalent to about 1.3 times the G2 + M + G1 period. As shown in Chart 3, the results judged by increased mitotic indexes were quite satisfactory.

The NR-FS tumor is an asynchronous cell population with a constant level of the mitotic index at the exponentially growing stage; however, the mitotic indexes after the last injection of ara-C appeared as 2 peaks (Chart 3). The first peak (lasting from Hr 5 to 8) is probably the result of effective blocking of the radiation lethality.
metaphase arrest agents in the point of dose and interval response (12). Although the sole injection of VBL (0.5 mg/kg body weight) had no effect on the TGD time or mouse survival time, the additional treatment with VBL to the ara-C-induced synchronized cohort of cells prolonged the TGD by 1.5 times (10.3:15.0) and the SD50 by 1.4 times (133.3:183.6) more than the ones caused by radiotherapy with simple ara-C synchrony. Madoc-Jones and Mauro (9) proved that in cultured mammalian cells VBL produced lethal effects during interphase (late G1 and S) at higher concentrations than those sufficient to induce mitotic arrest. Although administration of the relatively high degree of VBL used in our experiments would inactivate some of the nonsynchronized interphase cells, the side effects on normal tissue were not significant, since the mice thus treated survived longer than in any other case.

In this study, we have used exponentially growing tumors in which most of the cells were actively synthesizing DNA and dividing rapidly. Thus, ara-C and VBL treatment resulted in a high dose of synchronization. If the tumor's cell population contains a large proportion of noncycling cells, the degree of synchronization will be reduced. So the regimen studied in this experiment cannot be applied directly to most clinical cases, because virtually all tumor tissue contains a large noncycling cell population. The combination therapy of radiation and in vivo synchrony, however, might be more useful when the radiation is administered immediately after removal of the noncyclic hypoxic area of tumor tissue by surgery. We have performed intraoperative radiotherapy for abdominal cancer, especially for carcinoma of the biliary system, since November 1973, and this procedure increased the effectiveness and length of palliation for unresectable lesions (14). Recently, a combination of surgical resection with simultaneous intraoperative irradiation has been used for patients with noncurative resection of hilar bile duct and gall bladder cancer (5). A better prognosis may be obtained by the combination of intraoperative radiotherapy and in vivo synchronization.

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REFERENCES

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