Cidal and Subcidal Effect of Triethylene Thiophosphoramide on Cell Kinetics of Human Bladder Carcinoma Cells in Vitro

TETSURO KATO and ROBERT J. IRWIN, Jr.*

Department of Urology, Akita University School of Medicine, Akita and *Department of Urology, Massachusetts General Hospital, Boston

Tohoku J. exp. Med., 1977, 121 (4), 391-400 — In view of increasing topical use of various chemotherapeutic agents for bladder carcinoma, an experimental study concerning the effect of triethylene thiophosphoramide (Thio-Tepa) on cell kinetics of bladder carcinoma cells was performed, working on an established cell line of human bladder carcinoma. This polyfunctional alkylating agent, which is most widely used for instillation chemotherapy of bladder carcinoma, revealed a concentration dependent cytotoxicity against the cells. DNA precursor incorporation suggested that repair mechanism occurred following subcidal dose of this compound and faulty repair took place following cidal dose. The cell cycle was prolonged after subcidal treatment, the main effect being seen in DNA synthetic phase, and the changes in the cell cycle parameters returned to the normal within 2 cell cycle time. Repeated treatment with subcidal dose at an interval of 48 hr led to more extensive changes in the cell cycle as compared with that of single dose. Repeated exposures to subcidal dose, however, did not show any differences in the growth curves from those of the controls.

Intravesical instillation of chemotherapeutic agents is expected to be a valuable tool for the treatment of bladder carcinoma, for the cancerous tissues could be directly exposed to higher concentration of the agents with less toxicity as compared with systemic administration of the agents. A variety of agents have been employed topically and there is suggestion that some of the compounds lead to clinically beneficial effect on this disease (Bateman 1955; Veenema et al. 1962; Esquivel et al. 1965; Abassian and Wallace 1966; Riddle and Wallace 1971; Mishina et al. 1975). Veenema (1968) reviewed his results in over 100 patients with bladder carcinoma who were submitted to intravesical instillation of triethylene thiophosphoramide (Thio-Tepa) and stated that this therapy produced objective remission and even complete disappearances of superficial bladder tumors. One-third of his patients had complete tumor disappearance, one-third had partial regression and the remaining third had no effect. Since his report this polyfunctional alkylating agent has been widely used for topical chemotherapy.
of bladder carcinoma and general agreement with the clinical usefulness of Thio-
Tepa is established (Jones and Swinney 1961; Wescott 1966; Veenema et al.
1969).

There are, however, still many questions to be answered about the use of Thio-
Tepa for local treatment of bladder carcinoma. The urologist has no definite
idea as to whether Thio-Tepa could produce permanent cure or what the most
effective dose schedule is. Although Thio-Tepa instillation is best reserved for the
urologist at present as an adjunct to surgical treatment for bladder carcinoma,
specific information as to the effect of this compound on the bladder carcinoma
cell itself is insufficient. In view of increasing topical use of various chemother-
aputic agents for bladder carcinoma, an experimental study of the effect of Thio-
Tepa on cell kinetics was conducted. In the present work an established cell line
of human bladder carcinoma was employed as an in vitro model.

MATERIALS AND METHODS

Cell line and cell growth. An established cell line, MGH-U1 cells, was used in this
experiment. The cells were originally developed from stage D, grade 4 transitional bladder
carcinoma in the Massachusetts General Hospital, Boston, and have been successfully
subcultured since 1972. Appropriate number of the cells was planted on small round
cover slips (12 mm in diameter) and incubated in a humidified atmosphere of 5% 
CO$_2$-95% air mixture at 37°C with McCoy 5A modified medium supplemented by 10% 
fetal calf serum. After trypsinization viable cell number per cover slip was counted by the dye exclusion method and plotted in semilog-graph paper as a function of time.

$^3$H-thymidine incorporation. The precursor incorporation was measured by pulse-
labeling of the cells with 1.0 µCi/ml of $^3$H-TdR ($^3$H-methyl thymidine, specific activity 6.7 
Ci/mM, New England Nuclear Co.) dissolved in the medium. After 30 min of incubation, the cover slips on which the cells were grown were rinsed in phosphate buffer solution and fixed in acetic-ethanol (1: 3) solution for 15 min followed by rinsing in three changes of 70% ethanol and in distilled water. Subsequently the cells were treated with three changes of ice cold 3% perchloric acid for 15 min and rinsed in distilled water. The cover slip was transferred into a scintillation vial in which 1 ml of NCS solubilizer (Amersham Serle Co.) was added to digest the cells on the cover slip. Scintillation counting was performed on a Packard Tricarb model #2420 liquid scintillation counter with 20 ml of toluen POPOP scintillator.

Cell cycle analysis. The cells on the cover slips were pulse-labeled for 30 min with 0.1 µCi/ml of $^3$H-TdR, followed by washing in prewarmed medium containing 10⁻⁵ M of cold TdR 3 times, and reincubated in the same medium. Immediately following the pulse-labeling the cover slips were picked up every 2 hr through 36 hr. The cells on the cover slips were fixed and extracted in the same manner as described above. The cover slips were then attached on a glass slides with histolad and autoradiography was carried out by a dipping method using Kodak NTB-2 emulsion. After 2 weeks of exposure in dark-
dry condition at 4°C, the slides were developed with Kodak D-19 developer, fixed with 
30% thio-sulfate and stained with toluidine blue solution. The fraction of labeled mitoses (FLM) was calculated at each experimental time, so that the FLM curve was traced as a function of time. The FLM curves were analyzed according to Mendelsohn-Takahashi's asymmetry method (1971) which is a global type of cell cycle analysis.

Thio-Tepa experiment. The drug experiment started on day 3 of culture when the cells were in logarithmic proliferation. The cells were exposed for 2 hr to appropriate concentration of Thio-Tepa dissolved in the medium. After 2 hr the cells were thoroughly rinsed with 3 changes of prewarmed medium and reincubated in the drug-free medium.
When the cell cycle immediately after Thio-Tepa administration was concerned, $^3$H-Tdr was added 30 min before removal of the drug into the Petri dish in which the cover slips had been placed with the drug, so that both the drug and the precursor were washed off at the same time.

**RESULTS**

**Cytotoxic effect of Thio-Tepa**

Viable cell numbers after Thio-Tepa exposure for 2 hr at concentrations of 10 μg/ml, 50 μg/ml and 100 μg/ml, are plotted as a function of time in Fig. 1. It is found on the growth curves that 10 μg/ml of Thio-Tepa produces a temporarily static effect on the cell growth followed by exponential cell proliferation, 50 μg/ml results in more prolonged static effect and 100 μg/ml brings exponential cell death. Although the growth curves are not traced for more than 13 days in the figure, proliferation of the cells treated with 50 μg/ml is going to decline on the 10th day after the treatment. Actually no viable cells could be found in the culture 14 days after exposure to 50 μg/ml of Thio-Tepa. From these observations Thio-Tepa is considered to have concentration dependent cytotoxicity on the cells, indicating subcidal dose with 10 μg/ml and cidal dose with 100 μg/ml as well as 50 μg/ml under the condition of 2 hr exposure.

![Fig. 1. Growth curves of MGH-U1 cells exposed to Thio-Tepa (TTPA) for 2 hr. The cells were treated with 0 μg/ml (control ○), 10 μg/ml (▲), 50 μg/ml (●) and 100 μg/ml (□) on day 3 of culture. No viable cells were found 14 days after the treatment with 50 μg/ml and 100 μg/ml.](image-url)
Transition of $^3$H-TdR incorporation per unit cell number is illustrated in Fig. 2. The precursor incorporation by the untreated control cells increases exponentially during the logarithmic phase and then decreases rapidly to the low level in the stationary phase. The tidal dose of Thio-Tepa, 100 $\mu$g/ml as well as 50 $\mu$g/ml, causes a remarkable decrease of $^3$H-TdR incorporation and there occurs a temporary increase of the incorporation 3 or 4 days following the treatment. On the other hand, the static dose, 10 $\mu$g/ml, of the agent reveals a similarity of $^3$H-TdR incorporation with the control, while the relative count per cells of the treated cells is continually higher than the control during the logarithmic phase.

![Fig. 2. $^3$H-thymidine incorporation into the cells exposed to 10 $\mu$g/ml (---), 50 $\mu$g/ml (-----) and 100 $\mu$g/ml (-----) of Thio-Tepa on day 3. The cells were pulse-labeled for 30 min at the indicated points and sacrificed for scintillation counting.](image)

**Alteration of the cell cycle**

The cell cycle was analyzed on the cells exposed to 10 $\mu$g/ml of Thio-Tepa, because the number of mitoses was insufficient for the cells treated with tidal dose to be successfully traced in the FLM curve. The FLM curve immediately following the drug exposure is presented in Fig. 3. Prolongation of the cell cycle can be readily recognized as compared with the control, with the principal effect being seen in $S$ (DNA synthetic) phase duration. Moreover, the height and the trough of the FLM curve reach closely the same levels of the control, suggesting nearly all of the cells are affected by the agent.

Cell cycle parameters are summarized in Table 1, where $(T_i)$ is the transit time of $i$ phase calculated by Mendelsohn-Takahashi's asymmetry method. $T_c$, cell cycle time or generation time, of the treated cells is prolonged by approximately...
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Table 1. Cell cycle parameters of MGH-U1 cells immediately after the treatment with 10 μg/ml/2 hr of Thio-Tepa on day 3 of culture, analyzed by Mendelsohn-Takahashi's asymmetry method (1971)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thio-Tepa</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(T_c)_\text{asym. corr.}$</td>
<td>26.2 hr</td>
<td>34.7 hr (+32.4%)</td>
</tr>
<tr>
<td>$(T_o)_\text{area. corr.}$</td>
<td>8.9 hr</td>
<td>12.0 hr (+46.3%)</td>
</tr>
<tr>
<td>$(T_{G_1+G_2})_\text{est.level}$</td>
<td>3.6 hr</td>
<td>4.1 hr (+13.9%)</td>
</tr>
<tr>
<td>$(T_{G_1+G_2})_\text{est.level}$</td>
<td>14.4 hr</td>
<td>18.6 hr (+28.1%)</td>
</tr>
<tr>
<td>$(CV)_\text{est.}$</td>
<td>34.3%</td>
<td>41.8%</td>
</tr>
</tbody>
</table>

Increasing rate of the parameters is shown as per-cent of the control in the parenthesis.

30% as compared with the control. S phase is elongated by 46% of the control and $G_1$ phase is less effected than $S$ phase, while alteration of $G_2$ phase is much less than those of the former two phases.

As seen in Fig. 1 regrowth of the cells takes place within 2 days after the treatment with static dose of Thio-Tepa, being assumed that the cell cycle should be changed again. Cell cycle analysis was performed 48 hr following the treatment, when the cells were on day 5 of culture. The FLM curve of the treated cells, which is illustrated with a dotted line in Fig. 4, becomes nearly close to that of the control. Analysis of cell cycle parameters summarized in Table 2 confirms this observation; that is, $T_C$, $T_S$ and $T_{G_1}$ of the treated cells are rather shorter than those of the control, whereas $T_{G_2}$ remains unchanged.

What is interesting is that $T_C$ of the untreated cells on day 5 decreases to 21.9 hr which is clearly shorter than the value of 26.2 hr on day 3. Reduction of $T_C$ is calculated, to be attributed to decrease of $S$ and $G_1$ phase durations as presented in Table 2.
Fig. 4. The FLM curves on day 5. The cell cycle of the untreated control cells (---○---) is reduced as compared with that of day 3. The FLM curve 48 hr following the single exposure to 10 µg/ml of Thio-Tepa (---●---) is nearly close to the control, and the FLM curve immediately after the repeated treatment reveals again a prolongation of the cell cycle (10 µg+10 µg at 48 hr interval, double exposure ---×---).

Table 2. Cell cycle parameters of the cells on day 5 of culture, analyzed by Mendelsohn-Takahashi's asymmetry method (1971)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Thio-Tepa single*</th>
<th>Thio-Tepa double†</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(T_s)$ asym. corr.</td>
<td>21.9 hr</td>
<td>20.2 hr</td>
<td>32.4 hr (+47.9%)</td>
</tr>
<tr>
<td>$(T_s)$ area, corr.</td>
<td>6.6 hr</td>
<td>6.4 hr</td>
<td>10.2 hr (+54.5%)</td>
</tr>
<tr>
<td>$(T_{G_2+M})$ est. level</td>
<td>3.6 hr</td>
<td>4.5 hr</td>
<td>5.1 hr (+41.6%)</td>
</tr>
<tr>
<td>$(T_{G_1+M})$ est. level</td>
<td>11.7 hr</td>
<td>9.9 hr</td>
<td>17.1 hr (+46.1%)</td>
</tr>
<tr>
<td>$(CV)$ est.</td>
<td>32.7%</td>
<td>31.8%</td>
<td>39.5%</td>
</tr>
</tbody>
</table>

* Thio-Tepa single: The cells were treated with 10 µg/ml/2hr of Thio-Tepa on day 3 and reincubated for 48 hr in the drug-free medium.
† Thio-Tepa double: The cells were repeatedly exposed to the same dose on day 3 and 5 at 48 hr interval and the cell cycle was analyzed immediately after the 2nd treatment. Increasing rate of the parameters is presented in the parenthesis.

Effect of double exposure to Thio-Tepa on cell proliferation

The cells were repeatedly exposed to 10 µg/ml of Thio-Tepa at intervals of 12 hr and 48 hr following the first exposure on day 3. The growth curves are traced in Fig. 5, in which no distinctive differences between the treated groups can be detected. In other words, repeated doses of Thio-Tepa result in the same effect on the cell proliferation as that of single dose.

The FLM curve immediately following the second exposure reveals again prolongation of the cell cycle as illustrated with a broken line in Fig. 4 and the calculated parameters are presented in Table 2. Compared with the single dose effect, increasing rates of the cell cycle parameters are more extensive and unequity of alteration among the parameters becomes less prominent in the doubly treated cells (Tables 1 and 2).
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Fig. 5. Effect of repeated subcidal-dose of Thio-Tepa on cell proliferation. TTPA single —○--; exposed to 10 µg/ml/2 hr on day 3, TTPA double 12 hr —▲--; repeatedly exposed to 10 µg/ml/2 hr on day 3 at 12 hr interval, TTPA double 48 hr —×--; repeatedly exposed to 10 µg/ml/2 hr on day 3 and 5 at 48 hr interval. All of the experimented groups show a lag in the growth curve immediately after the treatment, but grow up thereafter to confluency.

DISCUSSION

The alkylating agents are the single most useful group of the cancer chemotherapeutic agents, even though they could not significantly prolong the life of patients in most malignancies. There has been much controversy as to whether basic differences exist between the various alkylating agents. Although a detailed pharmacologic discussion is beyond the scope of the present work, previous investigations have shown that the alkylating agents produce their effect by interstrand binding from the N7 in guanine of one DNA strand to the N7 of guanine on the opposite strand (Pullman and Pullman 1959; Brookes 1964; Lawley and Brookes 1965; Roberts and Warwick 1963). The interstrand binding of the polyfunctional alkylating agents prevents the separation of the two strands of DNA in double coiled helix necessary for cell replication, and thus inhibits cell proliferation in actively growing neoplastic cells.

The alkylating agents including Thio-Tepa have been reported to keep concentration dependent cytotoxicity against proliferating cells in general (Shimoyama 1973; Van Putten 1974; Freshney et al. 1975). Our results also reveal the same effect of Thio-Tepa on the human bladder carcinoma cells, indicating a subcidal effect with 10 µg/ml/2 hr and a cidal effect with 100 µg/ml/2 hr as well as 50 µg/ml/2 hr.
It has been indicated that $^3$H-TdR is incorporated into DNA with high specificity under the condition employed in the present experiment and the measurement of the radioactive precursor incorporation is useful in comparative study (Everhart et al. 1973; Ball et al. 1973). Following the subcidal dose of this compound, $^3$H-TdR incorporation rate is found to be higher than that of the untreated control. It is supposed that DNA synthesis is enhanced by either accumulation of the cells in DNA synthetic ($S$) phase or overgrowth of the cells accompanied by repair mechanism. Prolongation of $S$ phase is observed immediately after the treatment, but the changes in the cell cycle parameters go back to the values of the control within 48 hr, while the enhancement of the precursor incorporation continues for a longer period until the cell growth reaches the stationary phase. Crathorn and Roberts (1968) reported data to indicate that enzymatic repair of cross-linking by sulfer mustard occurs in mammalian cells as well as in bacteria. Therefore it is much likely that DNA repair takes place in the bladder carcinoma cells exposed to the subcidal dose of Thio-Tepa and the repair mechanism exists until the cells grow up at confluency.

There happens a temporary increase of $^3$H-TdR incorporation a few days following the exposures to 100 $\mu$g/ml/2 hr of this compound, when the cells are falling into exponential death. It has been suggested that the lethal damage of the alkylating agents is due to faulty repair after excision of cross-linked nucleotides rather than the prevention of DNA replication by interstrand cross-linking (Alexander 1969). If it is the case with the present study, a faulty repair would occur just before the cell population is exponentially killed by the Thio-Tepa.

The cell cycle is considered to vary with various extrinsic stimuli including temperature, chemical substances, ionizing irradiation and ultraviolet. Working on synchronous HeLa cells, Ohara and Terashima (1972) observed prolongations of $S$ phase as well as $G_2$ phase after a treatment with 0.5 $\mu$g/ml/1 hr of Mitomycin $C$. There are several data that indicate a change in the cell cycle of mammalian cells after irradiation (Kim and Evans 1964; Brown and Berry 1968; Hermens and Barendsen 1969; Frindel and Tubiana 1971). Frindel et al. (1970) reported a lengthening of cell cycle when NCTC sarcoma cells growing as an ascites tumor were irradiated by 250 rads. $S$ phase was about twice the normal after irradiation and by 48 hr the shape of the FLM curve seemed to be going back to normal. In the present study it is proved again that the cell cycle of the bladder carcinoma cells is elongated following the subcidal dose of Thio-Tepa and returns to normal by 48 hr, that is, within 2 cell cycle. It could be concluded that the recovery of the cell cycle begins at the 2nd cell cycle after the treatment.

Effect on the cell cycle parameters is seen principally in $S$ phase, secondly in $G_1$ phase and least in $G_2$ phase. Although there is no available evidence in the present study to distinguish a block from a delay in certain cell cycle phases, prolongations of $S$ phase and $G_1$ phase mean a pile-up of the treated cells in those cycle phases. Repeated treatment with the subcidal dose of this agent at an interval of 48 hr results in a more pronounced elongation of the cell cycle with
less differences between the increasing rates of the individual parameters, as compared with single treatment.

The changes in cell cycle parameters observed after the single or repeated dose of Thio-Tepa invite the speculation that responses of these cells to subsequent treatment might be altered as compared with the untreated or the single-treated cells. So far as the growth curves are concerned, however, repeated treatments with the subcidal dose at varying intervals do not lead to any differences from the controls. Further detailed investigation remains to be employed, since the subcidal effect of chemotherapeutic agents is of importance in the light of tumor recurrence, and information of cell kinetics might provide a possibility to achieve improved treatment schemes in cancer therapy.

References


