Abstract

Cells of the Morris hepatoma 7777 cultured in vitro have elevated levels of cytoplasmic thymidine kinase similar to those found in vivo. The cell-doubling time is slightly less than 24 hr. The addition of 5-fluorouracil to the culture medium (10 μg/ml) inhibits cell division, stimulates thymidine kinase production, and results in cell death which increases with time. Treated cells release significantly (p ≤ 0.01) more thymidine kinase into the culture medium than do untreated cells, and this effect can also be seen following a 60-min pulsed incubation of the cells with 5-fluorouracil. The total activity of thymidine kinase released is proportional to cell death and to the number of cells originally on the plate. These results suggest the possibility of monitoring tumor response to therapy in vivo by measuring serum levels of thymidine kinase.

Introduction

In previous work, we have shown that a thymidine kinase isoenzyme exists in human fetal tissue which differs from the adult form in electrophoretic mobility, heat sensitivity, pH optima, inhibition by dCTP, and the ability to utilize nucleotides other than ATP as phosphate donors (20). Further studies showed that human fetal thymidine kinase was derived from the cytoplasmic (high-speed supernatant) cell fraction and that the adult form was associated with mitochondria (1, 4). Moreover, these 2 enzymes are coded by different chromosomes (21). Increased cytoplasmic thymidine kinase indistinguishable from the fetal form has been found in human cancers, malignant human cell lines, and SV40-transformed human fibroblasts (4, 10, 18). Thymidine kinase activity in 23 human neoplasms was higher than that in matched control tissue in 21 of 23 instances (8). Multiple forms of thymidine kinase have been found in tumor tissues such as Yoshida sarcoma, Ehrlich ascites, Hepatoma AH 130, Sarcoma 180, and Morris hepatomas 7793 and 7794A but not in normal rat tissues (14). Thymidine kinase activity in 8 solid Morris hepatomas was significantly higher than the activity in normal rat liver (17). There was a rough correlation between tumor growth rate and thymidine kinase activity of 11 rat hepatomas; in all cases, tumor activity exceeded that of normal liver (7). Elevated thymidine kinase activity has also been reported in another series of 8 rat hepatomas; finally, sera from animals with advanced Morris hepatoma 7777 tumors had significantly higher serum levels of thymidine kinase than did sera from their corresponding controls (19).

These results were encouraging and led to several questions. Is thymidine kinase released from the tumor cells? If so, is it released from viable or dying cells? Is it possible to enhance enzyme release by drug intervention? How long is the enzyme present in sera? Can serum levels of thymidine kinase be correlated with tumor size? In an attempt to answer these questions, we studied the effect of 5-FUra on Morris 7777 hepatoma cells in tissue culture because 5-FUra has been shown to be effective against this tumor in vivo, the number of tumor cells could be easily quantitated, the media from a large number of tissue culture plates could be frequently sampled for activity, and the effects of the drug could be more clearly defined, allowing us to plan subsequent animal studies. Moreover, study of these cells in tissue culture would allow us to determine whether the thymidine kinase was being released from viable or from dead and dying cells.

Materials and Methods

We obtained an established cell line of Morris hepatoma 7777 from Dr. T. H. Wepsic at the Veterans Administration Medical Center in San Diego, Calif. The cells require minimal essential media with 5% fetal calf serum and 2 mM glutamine and are incubated at 5% CO2 at 37°C. We have maintained these cells in tissue culture and shown that they have a doubling time just under 24 hr; moreover, cultured cells produce tumors when injected back into Buffalo rats.

A number of tissue culture experiments were performed which are outlined in more detail in the appropriate section under "Results." Briefly, cells were harvested, placed on culture plates, and studied when they were established and had reached a concentration of 1.0 to 1.5 million cells/plate. The number of intact cells was determined by harvesting plates and counting the cells in a hemocytometer. Cell viability was determined by counting those cells which retained the ability to exclude trypan blue in a hemocytometer.

5-FUra was added to a number of plates to produce a concentration of 10 μg/ml (7.7 × 10^6 mM solution). This concentration was chosen because it approximates the serum level following a standard patient dose. Our standard assay for thymidine kinase was performed in a total volume of 100 μl. The reaction mixture consisted of 20 μl containing 1 mM ATP, 5 mM MgCl2, and 2.5 μCi [methyl-3H]thymidine (Schwarz/Mann, Orangeburg, N. Y.; specific activity, 56 Ci/mmol). Aliquots of 70 μl of media from the cell cultures were added to the reaction mixture and brought to final volume with 0.05 M Tris-HCl, pH 7.4. The final mixture was incubated for 30 min at...
37° and spotted on a 2.4-cm-diameter disc of Whatman DEAE paper (Scientific Products, Irvine, Calif.). The disc was washed 3 times with 10 ml of 1 mM ammonium formate to remove unreacted substrate and once with 5 ml of methanol. After drying, the disc was placed in 5 ml of toluene/2,4'-tert-butylphenyl)-1,2,4-oxadiazole (New England Nuclear, Boston, Mass.) and counted in a Searle analytic liquid scintillation counter (Amersham/Searle Corp., Arlington Heights, Ill.). In the concentrations used, 5-FUra had no measurable effect on the enzyme assay.

The amount of thymidine kinase released in the media under the same conditions varied from study to study. This variation may have reflected cells in a different stage of passage or other unknown variables. Because of this variability, a separate control set of cells was included in each study.

Results were expressed as cpm/ml. Results were not expressed as cpm/ml/mg of protein because no purification steps were undertaken and, with 5% fetal calf serum in the media, results expressed as cpm/ml/mg of protein would have been meaningless.

RESULTS

Effect of 5-FUra on Cell Number in Vitro and Release of Thymidine Kinase into Media. We initially compared the effect of 5-FUra on the release of thymidine kinase from 7777 cells in tissue culture to the release of enzyme from untreated tumor cells. 5-FUra was added to 10 plates containing from 1.0 to 1.5 million cells each to produce a concentration of 10 µg/ml. Ten plates of untreated cells served as controls. Sample volumes of 70 µl from each plate were assayed for thymidine kinase activity at 12, 24, and 48 hr. At 12 hr, there was minimal thymidine kinase released into the media (480 cpm for control cell versus 474 cpm for the 5-FUra-treated cells). By 24 hr, however, mean enzyme activity in the media of the 5-FUra-treated cells was significantly greater than that of controls, 7200 and 500 cpm, respectively (p ≤ 0.01); and by 48 hr, the mean thymidine kinase activity in the 5-FUra-treated cells had increased to 12,400 cpm compared to 3,500 cpm for the controls (p ≤ 0.01). In this study, control cells had approximately 4 to 7 million cells/plate at 48 hr. At that cell concentration, clusters of cells were beginning to pile up on the plate, there was considerable debris in the media, and cell death occurred, as determined by trypan blue. Cell death due to overcrowding probably accounts for the thymidine kinase in the media of the control plates at 48 hr. We repeated the study (5 plates/data point) with the purpose of obtaining accurate cell counts at 12, 24, and 48 hr; the results showed a doubling time for the control cells of just less than 24 hr while the number of 5-FUra-treated cells remained relatively constant and then decreased steadily (Chart 1). There was little thymidine kinase released into the media by the healthy growing cells at 48 hr compared to the cells treated with 5-FUra.

Cell viability was measured by lack of dye uptake when stained with trypan blue. Approximately 70% of the intact 5-FUra-treated cells excluded trypan blue at 24 hr whereas only 40% excluded this dye at 48 hr. In contrast, the control cells retained more than 95% viability during the course of these experiments unless the plates became overgrown.

We performed a similar study using normal fibroblasts from Buffalo rats. Three separate experiments were performed with culture inoculations of 1.5 x 10^5, 2.5 x 10^5, and 4.6 x 10^5 cells/plate. One set of 3 cultures was carried as the control and the one set was treated with 5-FUra. After 24 hr of incubation at 37°, the 3 control plates contained 2.9 x 10^5, 3.7 x 10^5, and 7.0 x 10^5 cells, respectively. After 24 hr of exposure to 5-FUra, the corresponding plates contained 1.6 x 10^5, 3.4 x 10^5, and 3.9 x 10^5 cells. Thus, in the 5-FUra-treated cells, there was inhibition of cell division. There was no detectable thymidine kinase activity in the media of control or 5-FUra-treated cells. The experiments are not strictly comparable with tumor cells, since the fibroblast is much larger than the hepatoma cell, does not pile up in tissue culture, and yields fewer cells per plate by a factor of 2 to 6. Moreover, fibroblasts are fully confluent at 8 to 9 x 10^6 cells/plate and show contact inhibition. Hepatoma cells are confluent at 5 to 8 x 10^6 cells/plate with less evidence of contact inhibition. Fibroblasts appear to be much less sensitive to 5-FUra in vitro than the hepatoma cells.

In a similar study, 10 plates containing equal numbers of untreated and 5-FUra (10 µg/ml)-treated hepatoma cells were incubated for 24 hr and then harvested. The cells were washed and sonicated. The cellular debris was spun down, and the supernatant was assayed to determine the protein content and thymidine kinase activity of the growing cells. The 5-FUra-treated cells had approximately twice the quantity of protein (3.0 x 10^-7 mg of protein per cell) as did the control cells (1.4 x 10^-7 mg of protein per cell) and 4 times the thymidine kinase activity, 7.9 ± 1.7 cpm/cell compared to 2.0 ± 0.8 cpm/cell for the control cells. It should be noted that total protein in the control and 5-FUra-treated cells at 24 hr was similar since there were approximately twice as many control cells as 5-FUra-treated cells. These data support the conclusion that 5-FUra stimulated the production of thymidine kinase and inhibited cell division.

Pulsed Administration of 5-FUra on Release of Thymidine Kinase. Following 5-FUra injection in patients, the drug is
Cells at 24 Hr. Separate studies were performed 2 weeks after addition of 5-FUra. Studies described earlier showed that 5-FUra-treated cells contained approximately 7.9 cpm of thymidine kinase per cell at 24 hr. This value was multiplied by the number of dead cells to determine the maximum amount of thymidine kinase potentially released into the media. The actual release was determined by subtracting the background activity from the control plates. The results show a direct correlation between the original number of cells on the plate and subsequent thymidine kinase activity in the media (Chart 3).

Enzyme Stability in Tissue Culture. Since we were sampling media for thymidine kinase activity, it was necessary to test the stability of the cytoplasmic enzyme (the form of the enzyme elevated in the Morris hepatoma 7777 tumor cells) in tissue culture media. We homogenized several plates of tumor cells in culture media, spun the supernatant back into the media of cell-free tissue culture dishes, and incubated the plates at 37° under 5% CO₂. Aliquots were taken at 15 min, 1, 2, 4, 6, 7, 9, 10, 12, and 24 hr and assayed for thymidine kinase activity (Chart 2). Over 80% of the original activity remained at 12 hr and 50% was still present at 24 hr.

Thymidine Kinase Potentially Released by 5-FUra-treated Cells at 24 Hr. Separate studies were performed 2 weeks apart. In each study, cells were allowed to grow on the culture plates until they reached a concentration of approximately 1 million cells/plate, and then 5-FUra was added to produce a concentration of 10 μg/ml. Twenty-four hr later, the number of live and dead cells was determined by staining the cells with trypan blue and counting them in a hemocytometer. Studies described earlier showed that 5-FUra-treated cells contained approximately 7.9 cpm of thymidine kinase per cell at 24 hr. This value was multiplied by the number of dead cells to determine the maximum amount of thymidine kinase potentially released into the media. The actual release was determined by assaying a sample of the media. The results (Table 1) showed that 22 to 31% of the thymidine kinase potentially released into the media was present at 24 hr. The fact that we did not recover 100% of the enzyme potentially released can be partially explained by enzyme inactivation (Chart 2) and the possibility that some thymidine kinase may remain trapped in the dead cells.

Release of Thymidine Kinase into Media Proportional to the Concentration of 5-FUra. The concentration of 5-FUra (10 μg/ml) used in these studies was chosen because it approaches the plasma level that would be found in patients immediately following a standard patient dose. We studied the effect of other concentrations of 5-FUra on thymidine kinase release at 24 hr, and the results of this study (5 plates/data point) are presented in Chart 3. Up to 20 μg/ml (15.4 × 10⁻⁷ mM), there is increasing thymidine kinase released corresponding to increasing 5-FUra concentration.

Release of Thymidine Kinase as a Function of Cell Mass. To determine if thymidine kinase release was associated with cell mass, cells were grown to 0.8, 1.0, 1.5, 2.0, and 2.5 million cells/plate; there were 5 plates/data point. One series of plates was incubated with 5-FUra (10 μg/ml) for 24 hr while a second series of plates served as untreated controls. All plates were assayed for thymidine kinase at 24 hr, and the background activity from the control plates was subtracted from the activity in the corresponding 5-FUra-treated plates. Thymidine kinase activity in the media of control cells after 24 hr of incubation ranged from 500 to 1000 cpm in proportion to the number of cells on the plate. The results show a direct correlation between the original number of cells on the plate and subsequent thymidine kinase activity in the media (Chart 4). Additional studies have confirmed this observation.

Clearance of Thymidine Kinase from Serum. Detection of thymidine kinase activity in the serum of 5-FUra-treated mice was rapid. Based on these data, we planned experiments to determine if the cellular release of thymidine kinase required the continuous presence of 5-FUra.

We used 30 plates with approximately 1.5 × 10⁶ cells each. Ten plates were controls; 10 plates contained 5-FUra (10 μg/ml) for 1 hr, at which time the media containing 5-FUra were removed and fresh media without 5-FUra added; the final 10 plates were incubated with media containing 5-FUra (10 μg/ml) for 24 hr. All plates were assayed at 24 hr. Media from control plates had a mean thymidine kinase activity of 1200 cpm; media of cells incubated with 5-FUra for 1 hr had a mean activity of 4600 cpm and media from cells incubated with 5-FUra for 24 hr had a mean activity of 7800 cpm. Other experiments using incubation times of 0.5, 1, and 2 hr with 5-FUra (20 μg/ml) gave comparable results.

Thymidine Kinase activity in the media of control cells after 24 hr of incubation ranged from 500 to 1000 cpm in proportion to the number of cells on the plate. The results show a direct correlation between the original number of cells on the plate and subsequent thymidine kinase activity in the media (Chart 4). Additional studies have confirmed this observation.

**Table 1** Percentage of recovery of thymidine kinase potentially released by dead cells at 24 hr.

<table>
<thead>
<tr>
<th>Study</th>
<th>cpm 24 hr post-5-FUra</th>
<th>Potential cpm released (10⁶)</th>
<th>Total cpm in media at 24 hr (10⁹)</th>
<th>% of potential cpm recovered</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7.7</td>
<td>3.03</td>
<td>2.37</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>10.7</td>
<td>2.3</td>
<td>1.82</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Dead cells were defined by the inability of the cells to exclude trypan blue.
*Cells incubated with 5-FUra (10 μg/ml) contain 7.9 ± 1.7 cpm/cell at 24 hr.

**Chart 3.** Increasing concentrations of 5-FUra in the culture media up to 20 μg/ml resulted in increased release of cellular thymidine kinase activity at 24 hr.
thymidine kinase in the sera of tumor-bearing animals will depend on a number of factors, one of which is the clearance of the enzyme from sera. To evaluate this variable, we sonicated several plates of hepatoma cells and prepared a concentrated supernatant which assayed at approximately $2 \times 10^7$ cpm/ml. Each of 4 rats received a 0.5-ml injection i.v. at Time zero. Serum was obtained for assay before injection and at 15 min and 1, 2, 4, 6, 12, and 24 hr postinjection. The activity at 15 min minus the background at Time zero before injection averaged $44 \times 10^3$ cpm/10 μl of sera. This value was taken to be 100%, and the decrease in serum activity was plotted against time (Chart 5). The results from all 4 rats showed good agreement with only 12% of the original activity remaining at 12 hr. Apparently, thymidine kinase is cleared from the sera or inactivated relatively quickly.

**DISCUSSION**

A number of tumor-associated isoenzymes have been described with properties distinct from the isoenzymes present in normal adult tissues (6, 15, 16, 20). These tumor-associated isoenzymes are frequently found in fetal tissue, and their reappearance in the adult carcinoma is believed to result from depression of portions of the genome during oncogenesis or rapid growth (13, 15, 20).

We have demonstrated that one of these tumor-associated isoenzymes, thymidine kinase, exists in human fetal tissue, human carcinomas, malignant human cell lines, and SV40-transformed human fibroblasts and, furthermore, that it differs in electrophoretic mobility, substrate inhibition, and temperature sensitivity from the isoenzyme normally dominant in adult tissues (4, 10, 18, 20). We have also shown previously that the thymidine kinase found in the Morris hepatoma 7777 can be distinguished from the thymidine kinase present in the normal rat liver and that these differences parallel those present in the human tissues (19). Moreover, our results showed that tumor-bearing rats had elevated serum levels of thymidine kinase compared to controls and that these serum elevations were probably due to the cytoplasmic form of the enzyme released from the tumor.

In tissue culture work with the 7777 hepatoma, we demonstrate that pulsed incubation with treatment doses of 5-FUra inhibits cell division, increases the thymidine kinase activity, and results in cell death and release of thymidine kinase into the media; moreover, the activity of thymidine kinase is directly proportional to the number of cells originally present on the plate. In our studies, 25 to 30% of the 5-FUra-treated cells were no longer viable at 24 hr, and cell death increased to 60% by 48 hr. The increase in thymidine kinase activity in the media with time was clearly proportional to the increase in cell death.

The question then arises whether or not the increase in thymidine kinase activity in the media could be secondary solely to the induction of thymidine kinase in the 5-FUra-treated cells. Our studies showed that cells treated with 5-FUra released twice as much protein per cell at 24 hr as did the untreated controls; however, there were twice as many control cells at 24 hr as there were 5-FUra-treated cells; hence, the total protein released by sonications of both groups of cells was essentially the same. There was twice the thymidine kinase activity per mg of protein in the 5-FUra-treated cells compared to controls and for that reason we would expect twice as much thymidine kinase in the media of the 5-FUra-treated cells compared to controls if the increase in enzyme activity were simply due to the thymidine kinase induction by 5-FUra. In fact, the media of the 5-FUra-treated cells contained 6 to 14 times as much thymidine kinase as did the media of control cells, clearly indicating that dead and dying tumor cells release thymidine kinase into media far in excess of what would be expected simply by enzyme induction. Furthermore, these data suggest the possibility of measuring the serum levels of thymidine kinase to detect animals with tumors and to monitor response to therapy.

One potential problem which may occur when in vivo studies are initiated is the release of thymidine kinase from normal cells of rapidly proliferating tissues such as the gastrointestinal tract and bone marrow. Release of thymidine kinase from these tissues did not produce a significant problem in our previous study measuring serum levels of thymidine kinase in tumor-bearing rats (19). Whether or not release of thymidine kinase from these tissues would be a problem following 5-FUra administration in vivo remains to be evaluated. Additionally, other chemotherapeutic agents might well result in different patterns of cell death and thymidine kinase release. These agents would have to be investigated individually. Our purpose in this paper

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**Chart 4.** Cellular release of thymidine kinase at 24 hr was proportional to the number of cells on the plate at the time 5-FUra was added to the media.

**Chart 5.** A bolus of partially purified thymidine kinase was injected i.v. into each of 4 rats, and serum samples were obtained for enzyme activity at various time periods postinjection. Only 12% of the original activity remained 12 hr postinjection.
was to gain preliminary data in cell culture to show that the concept was feasible and to provide a base line for subsequent studies of an animal tumor known to be sensitive to 5-FUra.

TTP is required for DNA synthesis. Normally, TTP is derived from dTMP which in turn is derived from dUMP. Deoxythymidine is a breakdown product of DNA, but thymidine kinase catalyzes the transfer of a phosphate group from ATP or another nucleotide triphosphate to form dTMP, restoring thymidine to the pool of DNA precursors. For this reason, thymidine kinase has been considered a ‘‘salvage’’ pathway for DNA synthesis, but the presence of 2 forms of thymidine kinase, a cytoplasmic form coded by a gene on chromosome 17 of the human nucleus and a mitochondrial form coded by a gene on chromosome 16 (21), coupled with elevated thymidine kinase activity (particularly cytoplasmic activity) in fetal tissues, many tumors, and rapidly replicating tissues suggests that the enzyme plays a much more important role than that of merely returning thymidine to the pool of DNA precursors.

The enzymatic assay is cumbersome and, without further modification, does not distinguish between the mitochondrial form of the enzyme which appears to be normally present in the sera (unpublished results) and the cytoplasmic form associated with various tumors (19). To rapidly distinguish between the 2 enzymes and to use this approach clinically, it will probably be necessary to develop an immunological technique to detect thymidine kinase in the sera. Such a technique should considerably increase sensitivity and specificity. Progress in purifying the enzyme and in developing immunological techniques has already been made (2, 5, 9, 11, 12). In future studies, we plan to develop an immunoassay for cytoplasmic thymidine kinase and to conduct animal studies based on the tissue culture work presented here.

REFERENCES

Effect of 5-Fluorouracil on the Release of Thymidine Kinase from Hepatoma Cells \textit{in Vitro}

Andrew Taylor, Jr., Oliver W. Jones and Mary Anne Grishaver


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