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EARLY TEMPORAL CHANGES IN THE URIDINE KINASE ISOZYME PROFILE OF THE NOVIKOFF HEPATOMA IN RESPONSE TO 5-AZACYTIDINE TREATMENT

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Abstract—Using a protocol conducive to the development *in vivo* of tumor resistance to 5-azacytidine, it is shown that the adult (I) and embryonic (II) forms of uridine kinase in Novikoff ascites tumor cells undergo early transient, but marked increases in activity, before falling below control values. The activities of I and II reversibly increase approximately 2-fold within one and four generations, respectively, from the beginning of treatment, then decline to approximately 30 per cent of the control value by the tenth generation; II predominates between generation two and ten. These early fluctuations in the concentrations of I and II, which probably account in part for the reported initial non-correspondence between uridine kinase activity and degree of resistance to pyrimidine analogs, are relevant to the problem of the development of tumor resistance to pyrimidine analogs.

Earlier studies [1, 2] indicated that tumors with a relatively rapid growth rate possess two forms of uridine kinase (ATP–uridine phosphotransferase, EC 2.7.1.48), which are separable under defined conditions on Sepharose 6B columns. Species I and II, with molecular weights estimated to be 120,000 and 30,000, appeared to represent the respective adult and embryonic forms of the enzyme. Also species II was more heat stable than I under the specified conditions. The existence of two forms of uridine kinase in rapidly growing tumors may have particular relevance to cancer chemotherapy. For example, there appear to be subtle changes in heat stability and related properties of uridine kinase in tumor cells upon the development of resistance to 5-fluorouracil [3] and to 5-azacytidine [4]. It was, therefore, of interest to examine the differential changes in uridine kinase isozymes during the development of tumor resistance to these and other analogs requiring this enzyme for activation. In an initial study [5] designed to examine this parameter, a transient 3-fold rise in uridine kinase II was observed in S-37 ascites tumor cells carried in mice nine generations after initiation of chronic treatment with low doses of 5-azacytidine; at this time, the tumor cells had developed significant resistance to the analog. However, the total uridine kinase activity later dropped to 25 per cent of the pre-induced (control) level by the twentieth generation after initiation of treatment. Early changes in the ratio of the uridine kinase isozymes are of particular interest, since it is during this period that the development of resistance may be reversible.

The present communication describes the temporal variation in the two uridine kinase isozymes in Novikoff hepatoma ascites cells over ten generations of treatment of the tumor-bearing rats with low doses of 5-azacytidine. This rat hepatoma system was selected for more detailed studies of this problem because of its reproducibility with respect to uridine kinase activity profiles, high yield of tumor cells and an apparent lower toxicity of 5-azacytidine as compared to the murine model.

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MATERIALS AND METHODS

Tumor model. Novikoff ascites tumor cells, with a transplant generation time of 7 days, were carried intraperitoneally in 140–160 g female rats of the Sprague–Dawley strain (Laboratory Supplies, Indianapolis, Ind.). The tumor cells were either employed in experiments or reinoculated (2 ml of a 1:3 dilution of packed cells) on the seventh day post-transplantation. Where indicated, the tumor-bearing animals received three weekly doses (1 mg/kg body weight) of 5-azacytidine via the intraperitoneal route on days 2, 4 and 6 of each transplant generation to induce tumor resistance. Due to its instability, the 5-azacytidine solutions were prepared immediately before use.

Fractionation of the uridine kinase. Preliminary to column fractionation, the uridine kinase was partially purified from the cytosol of the Novikoff hepatoma ascites cells by use of streptomycin sulfate and ammonium sulfate, as previously described [1]. The 30–50% ammonium sulfate fraction of the cytosol was dialyzed twice for 3 hr against 50 mM Tris–HCl buffer, pH 7·4, containing 20% glycerol (v/v) and 20 mM mercaptoethanol prior to chromatography. An aliquot of this dialyzed preparation equivalent to 10 mg protein was applied to a 0·635 cm^2 × 56·0 cm Sepharose 6B column; the latter was equilibrated and the applied protein was eluted into 1·0-ml fractions with a medium containing 200 mM Tris–HCl buffer, pH 7·5, 2·0 mM mercaptoethanol and 20% (v/v) glycerol.

Uridine kinase assay. The standard assay [1] contained 150 mM Tris–HCl (pH 7·5), 10 mM mercaptoethanol, 10 mM $MgCl_2$, 1·0 mM ATP, 0·4 mM [2-^{14}C]uridine (0·03 μCi) and 100 μl of the enzyme preparation in a final volume of 200 μl. After incubating for 20 min at 37°, the reaction was ter-

minated by heating to 90° for 3 min. The denatured protein was removed by centrifuging and the supernatant was filtered through DEAE-cellulose discs (H. Reeve Angel and Co. Inc., Clifton, N. J.). After washing with water and drying at 60°, the discs were counted in liquid scintillant. The rate of uridine phosphorylation was linear over the time period studied. The activity of uridine kinase in each column fraction, or in the total eluates, is expressed as nmoles of uracil nucleotides (UMP, UDP and UTP) formed per 60 min at 37° per 100 μl of each 1·0-ml fraction from a column loaded with 10 mg protein from the 30–50% ammonium sulfate fraction. It should be noted that uridine kinase I is unstable at 37° in the absence of substrate [1], but is completely stable for at least 30 min at this temperature under the conditions of assay, i.e. in the presence of uridine.

Reproducibility of column profiles. The reproducibility of the Sepharose 6B profiles to within a standard error of 10% was demonstrated earlier [5]. Where the maximum heights to peaks I and II are recorded, corrections have been made for very slight peak overlap at their centers by assuming peak symmetry.

Assay of resistance. Seven days after the inoculation of the tumor cells, rats bearing control Novikoff hepatoma cells (sensitive line), or cells exposed to chronic treatment with 5-azacytidine (1 mg/kg intraperitoneally on days 2, 4 and 6) for five transplant generations (resistant line), were divided into two groups of three rats each. One group received saline (controls), while the second group received a therapeutic dose (3 mg/kg) of 5-azacytidine before removal of the cells for estimation of the rate of DNA synthesis *in vitro*. At 2 and 3 hr after administration of 5-azacytidine, 2 ml of ascitic fluid was removed from control and treated rats and the cells were collected, then washed in Krebs–Ringer phosphate, pH 7·4, by centrifugation. After resuspending the cells in Krebs–Ringer phosphate, they were adjusted to a uniform concentration of $2{\cdot}5 \times 10^7$ cells/ml. The incubation mixture consisted of 2 ml of the cell suspension and 10 μCi [^{3}H]thymidine (20 Ci/mmole) in a final volume of 2·5 ml. After incubation at 37° for 20 min with gentle agitation, the cells were washed and the DNA was extracted for radioassay as previously described [6]. The specific radioactivity of the DNA, which was proportional to the rate of DNA synthesis, is expressed as cpm/10^7 cells.

Survival studies to test the resistance to 5-azacytidine of the Novikoff hepatoma cells from rats treated according to the above protocol involved the administration to rats carrying the sensitive and resistant lines of a therapeutic dose of the analog (3·0 mg/kg) on days 2, 3, 4, 5 and 6 after the inoculation of a standardized dose of tumor cells, i.e. 1×10^7 cells/mouse. Treatment was terminated on day 6 and the survival of control and treated rats was recorded.

RESULTS

The Novikoff hepatoma has two forms of uridine kinase, designated I and II, in order of their elution from a Sepharose 6B column [1]. The object of the present study was to monitor the differential changes in the concentration of these species during the early stages of development of tumor resistance to 5-azacytidine.

Shown in Fig. 1 are the Sepharose 6B profiles of uridine kinase activity derived from the Novikoff ascites cells following triweekly treatment of the tumor-bearing rats for one or four generations, respectively, with 5-azacytidine. The relative heights of the two peaks in the untreated cells are also indicated. Throughout the present communication, the data are based on activities in 100 μl of the 1·0-ml column fractions when the column is loaded with 10 mg of the 30–50% ammonium sulfate fraction of the respective cytosols. The most striking response to chronic treatment with 5-azacytidine is a 2·4-fold increase in species I in generation one and a 2-fold increase in species II in generation four. The total activities under each peak, after correction for slight overlap, were strictly proportional to the height of the peaks. The uridine kinase profiles in ascites cells from control rats and from those treated for one, two, four and nine generations were duplicated to within 10 per cent, using two different animals.

Figure 2 summarizes the temporal changes during 10 transplant generations, of the separate concentrations of species I and II in Novikoff hepatoma in response to chronic treatment with 5-azacytidine. The maximum (peak) activities were estimated from Sepharose 6B profiles such as those shown in Fig. 1. Also shown is the relative temporal change in the total uridine kinase activity present in an aliquot of the 30–50% ammonium sulfate fraction of the cytosol equivalent to 10 mg protein; the total activity represents the summation of the activity in each 100-μl aliquot of each column fraction. Thus, during the first generation of treatment, there is approximately a 2·4-fold increase in the total uridine kinase activity, due mainly to a marked (2-fold), but transient increase in the activity of species I. In the second generation, the total activity has dropped as a consequence of

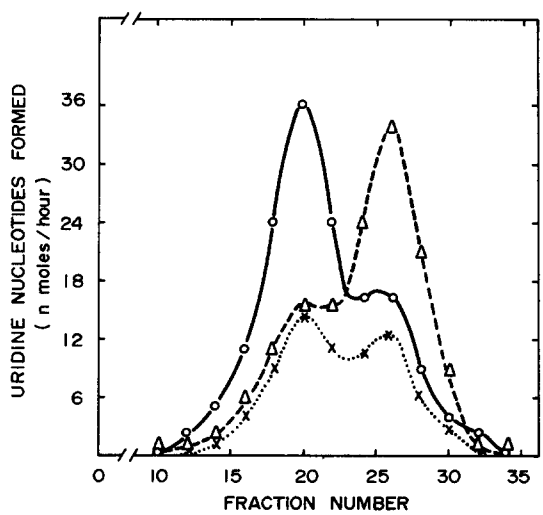

Fig. 1. Effect on the uridine kinase isozyme profile in Novikoff ascites tumor cells of triweekly treatment *in vivo* with 1 mg/kg of 5-azacytidine given intraperitoneally. Shown are the Sepharose 6B profiles based on 10 mg protein from the 30–50% ammonium sulfate fraction of the Novikoff cells one (—○—) and four (--△--) generations after initiation of 5-azacytidine treatment. Uridine kinase species I and II are centered at fractions 20 and 26 respectively. The relative concentrations of species I and II in Novikoff ascites cells from control rats are indicated (— × —) for comparative purposes.

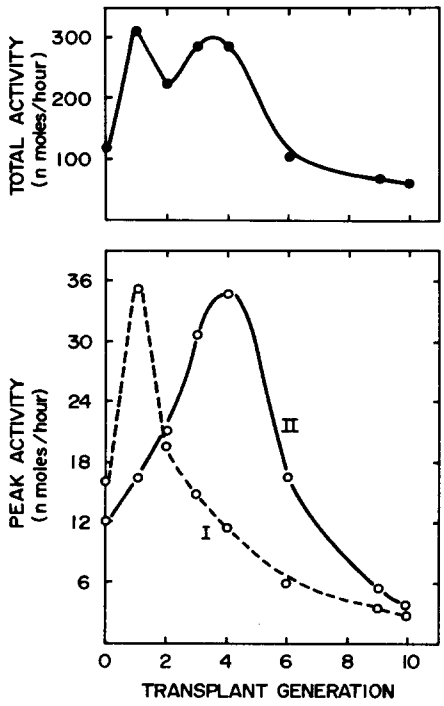

Fig. 2. Temporal changes in total uridine kinase activity (top) and in uridine kinase I (--○--) and II (—○—) in response to the chronic administration of 5-azacytidine over ten transplant generations. The activities shown were derived from the Sepharose 6B profiles.

the return of species I to the pre-induced level. However, throughout generations one to four there is a progressive rise in the concentration of species II, which becomes maximal in generation four and which accounts for a second temporary increase in total uridine kinase activity. Beyond their peak activities there is a rapid decline in both species I and II, and as a consequence, in total uridine kinase activity. It is of particular interest to note that between generations two and nine, uridine kinase II is the predominant species in the cell. Also, a significant difference was observed in both the K_m and V_{max} with 2-[^{14}C]uridine as substrate for species I and II, i.e. in three separate experiments the K_m for uridine kinases I and II (±S.D.) was 100 ± 12 and 54 ± 6 μM, while the V_{max} was 8·5 ± 1·1 and 4·6 ± 1·1 units respectively.

The reversibility of the 5-azacytidine-induced changes in the enzyme profile was tested by terminating treatment at the end of generation four, then determining the uridine kinase profile at the end of generation five. The results of this experiment indicated (D. E. Schumm, unpublished observations) that the isozyme profile was fully reversible (i.e. reverted to that of control tumor cells) within a period of one transplant generation.

The resistance of the growth of Novikoff hepatoma ascites cells from untreated (control) and 5-azacytidine-treated rats to a therapeutic dose of 5-azacytidine (3 mg/kg) was tested (see Materials and Methods for details). In general, tumor-bearing rats which either received no treatment or were treated for five generations with low doses of 5-azacytidine received either saline (controls) or a therapeutic dose of 5-azacytidine (3 mg/kg), and DNA synthesis was assayed 2 and 3 hr later. The rats receiving low doses of 5-azacytidine on days 2, 4 and 6 were given the therapeutic dose on day 7 of the fifth generation of treatment. [^{3}H]thymidine incorporation *in vitro*, which was linear over the time period studied, was used as an index of DNA synthesis. The results indicate (Table 1) that at 2 and 3 hr after administration of 3 mg/kg of 5-azacytidine there is a 67 and 32 per cent inhibition, respectively, of [^{3}H]thymidine incorporation into DNA in the Novikoff hepatoma cells from untreated rats. In contrast, the incorporation of [^{3}H]thymidine into the DNA of cells from animals, which were treated for five generations with a protocol designed to induce resistance to 5-azacytidine, was not inhibitied. Thus, by the fifth generation, it appears that the ascites tumor cells have developed a significant degree of resistance to 5-azacytidine. This resistance was not due to decreased uptake of the analog,

Table 1. Effect of 5-azacytidine therapy of rats bearing sensitive or resistant hepatoma cells on subsequent DNA synthesis or survival

Cell line*	5-Azacytidine (3·0 mg/kg/dose)	[^{3}H]thymidine† (% control ± S.D.)	Survival‡ (days ± S.D.)
I. DNA synthesis *in vitro*			
Sensitive	2 hr	33 ± 2	
	3 hr	66 ± 5	
Resistant	2 hr	109 ± 10	
	3 hr	100 ± 3	
II. Host survival			
Sensitive	none		13 ± 0·6
	5 days		20 ± 1·4
Resistant	none		12 ± 1·0
	5 days		13 ± 1·9

* The resistant cell line was produced by administering 1·0 mg/kg of 5-azacytidine three times per week for five transplant generations (i.e. 5 weeks).

† The uptake of [^{3}H]thymidine into DNA of hepatoma cells obtained from rats receiving a single therapeutic dose (3·0 mg/kg) of 5-azacytidine is expressed as per cent of sensitive or resistant controls which did not receive therapeutic dose of 5-azacytidine. The experiment was performed 7 days post-transplantation of the tumor cells. The results (per cent ± S.D.) are based on three rats per point. The average specific activities of the controls in the sensitive and resistant lines were 2510 and 1820 cpm/10^6 cells respectively.

‡ For survival studies, the control animals bearing sensitive and generation 5 resistant cells received no treatment; the animals in the corresponding treated groups received five daily therapeutic doses of 5-azacytidine before treatment was terminated. Each survival value (days ± S.D.) is based on five rats.

since the uptake of [^{14}C]5-azacytidine by cells of both sensitive and resistant strains was equal.

To confirm that the higher incorporation of [^{3}H]thymidine into the DNA of the resistant strain as compared to the sensitive strain after treatment with a therapeutic dose of 5-azacytidine was a reflection of the true rate of DNA synthesis and cellular proliferation, a survival study was carried out. (Although an increase in DNA polymerase has been reported [7] to occur in leukemic cells in response to 5-azacytidine treatment, it does not seem probably that this alone would lead to increased incorporation of [^{3}H]thymidine into DNA.) For the survival study, rats carrying sensitive or generation 5 resistant lines of the Novikoff hepatoma were divided into two groups, each consisting of five rats. One group (controls) received saline, while the second group received 3·0 mg/kg of 5-azacytidine on days 2, 3, 4, 5 and 6 of the fifth generation. The results, shown in Table 1, indicate that the tumor-bearing animals treated for five generations with low doses of 5-azacytidine exhibited significant resistance to therapeutic doses of the analog. Thus the rats bearing the sensitive cells survived 50 per cent longer than those bearing cells of the resistant strain.

DISCUSSION

The theroretical basis of the present study is that key enzymes (or other proteins) which mediate the effect of chemotherapeutic agents may exist in multiple molecular forms which change quantitatively during development and in tumors during tumor progression, or of more immediate relevance, progression under the selective pressure of chemotherapy. The uridine kinase system, which is essential for activation of pyrimidine analogs such as 5-azacytidine, was selected [5] for the initial test of this possibility, since an earlier study [1] indicated that the enzyme existed in at least two molecular forms in tumors with a relatively rapid growth rate. The study showed [5] that uridine kinase II is markedly and preferentially induced in S-37 mouse ascites cells which have become partially resistant to 5-azacytidine in response to biweekly treatment with the analog for nine generations (i.e. 9 weeks); however, the activity dropped to 25 per cent of the pre-induced (control) value by generation 20, despite the continued administration of 5-azacytidine.

In the present investigation, the fluctuation in uridine kinase isozymes in Novikoff hepatoma cells has been studied during the first ten generations of treatment of rats with 5-azacytidine. Although species I showed a transient increase in the first generation, species II attained a maximum two-fold increase in generation four and constituted the predominant form of uridine kinase between generations two and nine.

Since the cells exhibit marked resistance to 5-azacytidine by the fifth generation, it appears that the differential changes in the concentration of the uridine kinase isozymes may be related to the development of resistance. Furthermore, since species II is the more heat-stable form [1] with a lower K_m for uridine (present study), its predominance during the early stages of development of tumor resistance is compatible with the reported [8] appearance of a more heat-stable form of uridine kinase with a lowered K_m for uridine in 5-azacytidine-resistant mouse leukemic cells. Results to be presented elsewhere (N. Greenberg and T. E. Webb, submitted for publication) show, according to prediction, that isozyme I has a high affinity for 5-azacytidine but a low affinity for uridine, while the reverse is true for species II. The isozyme fluctuations may also account for the apparent non-correspondence between the level of uridine kinase in Ehrlich ascites cells and the appearance of resistance to 5-fluorouracil during the early stages of treatment [9].

Both the predictability and the reversibility of the early changes in uridine kinase isozymes may facilitate reversal, or at least temporary postponement, of the development of tumor resistance during chemotherapy. For example, should the two isozymes have different kinetic properties, it may be possible to increase the therapeutic index by substituting the pyrimidine nucleoside analog currently used in chemotherapy with another analog which has a higher affinity for or is metabolized more rapidly by the isozyme preferentially induced during the early stage of chemotherapy. A multitude of enzymes have been shown to exist in more than one isozymic form, the relative proportions of which change in an apparently irreversible manner during development, in response to neoplastic transformation, or during tumor progression [10, 11]. Such irreversible changes in enzyme or isozyme profile are also commonly observed during chemotherapy, particularly during the development of tumor resistance. For example, the development of resistance to pyrimidine analogs is most commonly accompanied by a marked fall in uridine kinase activity [12], although other mechanisms have also been identified [13]. The later irreversible nature of the loss of uridine kinase activity, which has also been observed in this study, or other such changes which characterize the later stages in the development of resistance, tend to emphasize the potential importance of the earlier, apparently reversible events.

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