ASPARTATE CARBAMOYLTRANSFERASE INHIBITION AND URIDYLATE TRAPPING RESULT IN A SYNERGISTIC DEPRESSION OF URIDINE TRIPHOSPHATE IN HEPATOMA CELLS

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1. Introduction

The induction of lethal UTP deficiency by uridylate-trapping sugar analogs [1] depends on a relatively low rate of pyrimidine nucleotide biosynthesis [2]. Increased activities of enzymes involved in the de novo synthesis of pyrimidine nucleotides have been demonstrated in a spectrum of hepatomas [3,4]. The resistance of solid hepatomas [5] and of ascites hepatoma cells to the action of D-galactosamine (GalN) has been explained by the efficient compensation of uridylate trapping by de novo synthesis resulting in only a limited depression of UTP [6]. Noncompensated uridylate trapping can be induced by the synergistic action of GalN and an inhibitor of de novo pyrimidine synthesis. A potent inhibitor of aspartate carbamoyltransferase (EC 2.1.3.2) has been introduced recently [7–10]. *N*-(Phosphonacetyl)-L-aspartate (PALA) has been synthesized by Collins and Stark [7] as a transition state intermediate analog of the aspartate carbamoyltransferase reaction and inhibits the enzyme in a number of cells and tissues [8,9]. PALA is a competitive inhibitor with respect to carbamoylphosphate and has a K_i of about 10^{-9} mol/l for the enzyme from mammalian cells [9,10].

This paper demonstrates the depression of de novo uridylate synthesis and UTP contents by PALA in ascites hepatoma cells. The depression of UTP by either PALA or GalN is insufficient, however, for a complete and irreversible inhibition of hepatoma cell growth in suspension culture. Only the combination of GalN-induced uridylate trapping with the blockage of de novo pyrimidine synthesis results in severe UTP deficiency and cellular death.

2. Materials and methods

2.1. *Chemicals and enzymes*

D-Galactosamine · HCl was purchased from C. Roth, Karlsruhe, FRG. *N*-(Phosphonacetyl)-L-aspartate (PALA) was obtained from Dr G. Stark, Department of Biochemistry, Stanford University School of Medicine, Calif. 94305, USA. Swim's medium S-77 and Swim's 67-G medium were from Grand Island Biological Company, Grand Island, NY, USA; the uridine, cofactors, and enzymes used in this investigation were from Boehringer Mannheim, FRG.

2.2. *Incubation and suspension culture of ascites hepatoma cells*

The transplantable ascites hepatoma AS-30D [11] was carried in female Sprague-Dawley rats, the tumor cells were collected and incubated as described previously [12]. Transplant generations 426–436 were used in the present study. AS-30D cells were grown at 37°C under CO_2/air (5/100) in primary suspension cultures on a gyratory shaker at cell concentrations between 2 and 10×10^8/l in Swim's 67 G medium supplemented with glutamine (2 mmol/l), sodium phosphate (2 mmol/l) and ascitic fluid (9/100 by vol.) sterilized by filtration of AS-30D ascites hepatoma fluid. Proliferation and growth were monitored by cell counts in a blood counting chamber and by measurement of the absorbance of the suspensions at 623 nm; the latter correlated linearly with the cell wet weight in the concentration range studied.

2.3. *Nucleotide analyses*

Our procedure for freezing and deproteinization of

the cells has been described [12]. Total acid-soluble uracil 5′-nucleotides (ΣUMP) were measured as UMP after snake venom phosphodiesterase hydrolysis [13]. Total adenine and guanine 5′-nucleotides (ΣAMP and ΣGMP) [13], UDP-glucose, UTP, UDP [14] and ATP [15] were assayed enzymatically.

3. Results

3.1. *Inhibition of de novo uridylate synthesis by PALA*

Incubation of AS-30D cells in the presence of GalN (0.5 mmol/l) elicited an increase of total uracil nucleotides (ΣUMP) at a rate of about 0.8 mmol × h^{-1} × (kg cells)$^{-1}$ between 1 and 4 h (fig.1). Inhibition of aspartate carbamoyltransferase by PALA resulted in a dose-dependent suppression of this GalN-induced stimulation of de novo pyrimidine synthesis. PALA alone caused only a minor decrease of ΣUMP when compared to control cells (fig.1). A complete suppression of the GalN-induced increase of ΣUMP by PALA (0.8 mmol/l) and a more pronounced decrease of ΣUMP induced by PALA alone were seen when the concentration of P_i in the incubation medium was lowered from 26 [12] to 2 mmol/l.

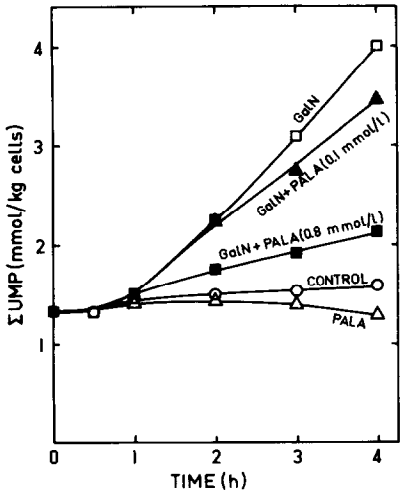

Fig.1. Changes in total acid-soluble uracil nucleotides (ΣUMP) induced by GalN and PALA. AS-30D cells were incubated in standard medium [12] at 37°C at cell concentrations of 1.8–2.4 × 10^9/l. The pH was kept between 7.45 and 7.25. Nucleotide contents are based on cell wet weights determined 3 times during the incubation [12]. Duplicate analyses from separate experiments under the same condition differed by less than 15%. The concentration of GalN was 0.5 mmol/l throughout; the concentration of PALA was 0.8 mmol/l (open triangles) or as otherwise indicated.

3.2. *Induction of selective UTP deficiency by noncompensated uridylate trapping*

The efficient compensation of the GalN-induced trapping of uridylate was indicated by the rate of increase of ΣUMP (fig.1) which was more than 2 times higher than in liver [1] and by the limited depression of UTP when the cells were incubated in the presence of GalN (fig.2). Inhibition of aspartate carbamoyltransferase led to a 60% reduction of the UTP content after 4 h (fig.2). This depressed UTP level still corresponds to the control range in rat liver [16]. The combination of uridylate trapping with the inhibitory effect of PALA resulted in a synergistic depression of UTP in these hepatoma cells to contents below 0.05 mmol/kg (fig.2).

The use of a low GalN concentration (0.5 mmol/l) enabled the induction of a selective UTP deficiency rather than a general depression of nucleotide contents seen with GalN concentrations above 1 mmol/l [12]. Neither PALA nor GalN nor combinations of both had any significant effect on the contents of total adenine (ΣAMP) and guanine nucleotides (ΣGMP) or ATP during the 4 h of incubation. These purine nucleotides remained within the control ranges determined earlier in AS-30D cells [12]. It was remarkable that the

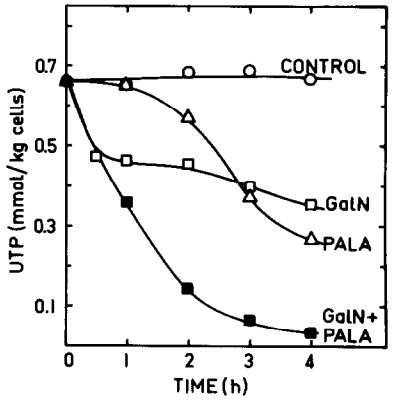

Fig.2. Depression of UTP contents induced by GalN and PALA. The experimental conditions were the same as described under fig.1. The concentrations of GalN and PALA were 0.5 and 0.8 mmol/l, respectively.

induction of UTP deficiency in these UDP-glucose-deficient cells [12] was not associated with a further depression of UDP-glucose which remained in a range of 0.03–0.07 mmol/kg.

Uridine (0.5 mmol/l) prevented or reversed the depression of UTP induced by GalN and PALA. UTP contents remained between 0.76 and 0.56 mmol/kg when AS-30D cells were incubated as described under fig.2 in the presence of uridine + GalN + PALA.

3.3. *Synergistic inhibition of cell growth in suspension culture induced by PALA and GalN*

A depression of the growth rate was observed when AS-30D cells were cultured in the presence of PALA or GalN (0.6 mmol/l each). A complete cessation of growth and proliferation of the cells was induced by the combined action of both drugs (fig.3). Examination of the cells after 23 h in culture by phase contrast microscopy and staining with trypan blue (final concentration 2 g/l) indicated more than 96% of intact cells in the controls and in cultures treated with either GalN or PALA. One third of the cells exposed to GalN + PALA took up trypan blue, cytoplasmic protrusions and/or cytonecrosis were seen in about 45%.

The addition of uridine (0.5 mmol/l) to the cultures (fig.3) after 25 h completely failed to restore growth or proliferation of cells treated with GalN + PALA

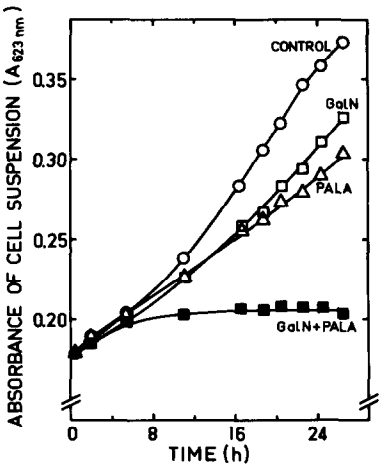

Fig.3. Depression of cell growth in suspension culture induced by PALA and GalN. Cultures were initiated at a concentration of 4×10^8 cells/l and growth was monitored by measurement of the absorbance of the suspension at 623 nm. The concentrations of PALA and GalN were 0.6 mmol/l each.

whereas cell growth continued in cultures grown in the presence of GalN or PALA. This indicated irreparable cell damage and irreversible loss of cell growth induced by severe UTP deficiency (< 0.05 mmol/kg, see fig.2) by 25 h.

4. Discussion

The limited depression of UTP contents in AS-30D cells induced by GalN (fig.2) is sufficient for triggering an increase in the rate of de novo pyrimidine synthesis. In previous work this increase was suppressed by the use of 6-azauridine [1,6,17] which leads to an inhibition of orotidine 5′-phosphate decarboxylase [18]. Inhibition of aspartate carbamoyltransferase by PALA [7,10] also causes a suppression of the GalN-induced increase in ΣUMP (fig.1). The available evidence indicates that de novo pyrimidine nucleotide synthesis in mammalian cells is regulated by feedback inhibition of glutamine-dependent carbamoyl-phosphate synthetase (EC 2.7.2.9) by UTP [19–21]. Lowering of the intracellular UTP concentration by GalN should markedly increase the accumulation of carbamoyl phosphate induced by an inhibition of aspartate carbamoyltransferase. This would provide further convincing evidence for the operation of the feedback inhibition of glutamine-dependent carbamoyl-phosphate synthetase by UTP in intact cells.

The induction of lethal UTP deficiency by the synergistic action of PALA and uridylate trapping may serve as a new approach to the chemotherapy of hepatomas. PALA, in contrast to 6-azauridine, allows a selective protection of the liver by means of orotate. Orotate pretreatment has been shown to prevent the liver injury caused by GalN-induced UTP deficiency [22]. As compared to liver, orotate uptake by a wide range of experimental hepatomas including the most slowly growing tumors, has been shown to be very low [23,24]. Orotate (0.5 mmol/l) also has no detectable effect on the UTP contents of AS-30D cells incubated in the presence or absence of GalN [17]. The concentrations of PALA required for inhibition of de novo pyrimidine synthesis (fig.1) and depression of cell growth (fig.3) in AS-30D cells are higher than the effective concentrations in transformed hamster cells (C13/SV) and mouse cells (SV 3T3) [10]. The toxicity of PALA in the latter cell lines is completely prevented

by addition of uridine to the culture medium [10].

The time period of severe UTP deficiency required for irreparable or lethal damage has recently been studied in AS-30D cells by uridine reversal of UTP deficiency induced by 6-azauridine + GalN [17]. This period ranges from 3–20 h [17]. It is consistent that uridine failed to restore cellular growth in the presence of PALA + GalN after 25 h corresponding to a UTP deficiency period of about 22 h (see fig.2). The demonstration of a synergistic action of PALA and GalN supports the usefulness of the concept of a noncompensated uridylate trapping that can be extended to other nucleotide-trapping analogs in combination with inhibitors of de novo nucleotide biosynthesis [17].

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