Mechanism and control of degradation and resynthesis of adenylates in tumour cells

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A comparative study revealed that Ehrlich ascites carcinoma (EAC) cells use glutamine plus inosine for regeneration of adenylates via the purine nucleotide cycle, whereas AS 30D hepatoma cells use adenosine instead. This observation can be correlated with the very low production of aspartate from glutamine in hepatoma cells. Although glucose is an important energy fuel for EAC, it cannot maintain a high enough level of adenylates unless glutamine is also present. Kinetic analysis of hydrolysis of ATP and ADP in the presence of rotenone suggests that deamination of AMP does not maintain a high enough ATP/ADP ratio and probably does not act as energy buffer after inhibition of cell respiration. It seems that, compared with normal cells, malignant cells have the ability for a very rapid regeneration of adenylates. It is proposed that instability of the adenine nucleotide pool, owing to frequent aerobic–anaerobic transitions, represents an essential feature of neoplasia, with profound impact on the whole metabolism of tumour cells.

INTRODUCTION

There is a large body of evidence showing that, owing to a high rate of proliferation which is not followed by an adequate vascularization, malignant tissues become ischaemic and consequently hypoxic or anoxic [1]. Permanent anoxia (or ischaemia) leads to cell death and necrosis of tumour tissue. It is likely, however, that before this occurs the cells suffer from intermittent anoxia (ischaemia) owing to a decrease in pO_2 , which becomes a limiting factor for cell respiration [2–4]. The occurrence of anoxia is inevitably followed by degradation of the pool of adenine nucleotides, whereas reoxygenation would bring about regeneration of the pool if the changes were reversible. Adenine nucleotides interact with most metabolic pathways in a living cell, and in general terms one could say that almost all cell metabolism is organized around adenine nucleotides [5]. We therefore assume that instability or imbalance of the pool could be one of the essential features of energy metabolism of malignant cells. For that reason the problem of instability of concentration of adenylates in cancer cells deserves greater attention, although it has been the subject of numerous investigations [6–11]. We reported recently [12] that the operation of the purine nucleotide cycle is an important mechanism for regeneration of the pool of adenine nucleotides in Ehrlich ascites carcinoma (EAC) cells on reoxygenation. It was shown also that alternations of aerobic and anaerobic conditions brought about a significant decrease in aspartate compared with the control and that this effect is absent in the presence of hadacidin, a specific inhibitor of adenylosuccinate synthetase. This finding suggested that the function of a high rate of glutamine oxidation via the aspartate aminotransferase pathway in EAC may be to support regeneration of adenylates. Transitions of the cells from the anaerobic to aerobic state, and vice versa, in the presence of glutamine plus inosine revealed that there are two components of the adenine nucleotide pool, one which is stable and the other which is variable and responds to the aerobic–anaerobic transitions. These findings raised new questions about the mechanism and regulation of degradation and resynthesis of adenylates in tumours.

The present work confirms and extends our previous study of the regulation of the adenine nucleotide pool in EAC cells [12].

Also, we report data obtained with AS 30D hepatoma which show that the cells of these two kinds of tumours have different mechanisms for resynthesis of AMP and very different capabilities for the oxidation of glutamine via the aspartate aminotransferase pathway.

MATERIALS AND METHODS

All the experiments were performed *in vitro* with EAC and AS 30D hepatoma cells. The cells were propagated in the abdominal cavity of Swiss mice (EAC) or Sprague–Dawley rats (hepatoma) and harvested usually 7–10 days after transplantation. They were mixed with ice-cold Krebs–Ringer solution [12] and filtered through four layers of cheesecloth. The cells were washed twice in the same solution. The details of experimental conditions and preparation of the cells were described elsewhere [12–14] and in the legends to Figures and Tables.

The cells were incubated at 30 °C in the same physiological solution, except that 20 mM-Tris/HCl and 20 mM-Mops, pH 7.4, were added in order to increase buffer capacity, which was important if the cells were incubated with glucose. Washed cells were kept as a thick suspension on ice (approx. 350 mg wet wt. of cells/ml). The amount of the cells was determined by measuring wet weight after centrifugation for 2 min in an Eppendorf 3200 centrifuge. All incubations, except when O_2 consumption was measured, were carried out in a Dubnoff metabolic shaker in 25 ml Erlenmeyer flasks, whereas anaerobic conditions were achieved by incubating the cells with N_2 or in plastic tubes completely filled with cell suspension and closed. The incubation mixture contained usually 130–150 mg wet wt. of cells/ml.

The integrity of fresh cells was tested by permeability to Trypan Blue and by measuring ATP concentration or the rate of cell respiration by a Clark oxygen electrode.

Concentrations of metabolites (adenine nucleotides, aspartate etc.) were measured by the standard enzymic methods [15]. All the experiments were repeated three to four times with different batches of the cells, and representative reproducible results are presented. The measurements are expressed per mg wet wt. of cells.

RESULTS

Effect of glucose, glutamine and inosine on the pool of adenine nucleotides under aerobic conditions and in the presence of rotenone

EAC cells were incubated aerobically for 30 min. After this time samples were taken for measurement of adenine nucleotides, and incubation was continued for the next 30 min in the presence of rotenone, which is an inhibitor of the respiratory chain (Site I). The data presented in Table 1 indicate that the addition of glucose together with inosine had no significant effect on the pool of adenine nucleotides. The concentration of ATP was virtually the same in the presence and absence of rotenone. Addition of glutamine plus inosine markedly increased the concentration of ATP and the total pool of adenylates, but addition of rotenone under these conditions decreased the level of ATP to an unmeasurable value. The total adenine nucleotide pool in the presence of rotenone was equal to that found in the control. It should be noted that, if glucose was added together with glutamine, inosine and rotenone, the total pool of adenylates was

Table 1. Effect of glucose, glutamine and inosine on the pool of adenine nucleotides in EAC cells incubated aerobically and in the presence of rotenone

The cells were incubated for 30 min aerobically and then for 30 min in the presence of rotenone in the standard Krebs–Ringer phosphate medium, pH 7.4. After incubation with rotenone, the pH of the incubation medium was measured. Additions: 20 mM-glucose, 8 mM-glutamine, 1 mM-inosine, 3.3 µg of rotenone/ml and 163 mg of the cells/ml. Abbreviation: AdN, total pool of adenylates.

Experimental conditions	pH	Adenine nucleotides (nmol/mg of cells)			
		ATP	ADP	AMP	AdN
Control	7.35	1.81	0.20	0.38	2.40
Glucose aerobic		1.66	0.18	0.52	2.35
+ rotenone	6.40	1.60	0.25	0.58	2.42
Glucose + inosine aerobic		1.94	0.22	0.42	2.58
+ rotenone	6.35	1.85	0.23	0.54	2.62
Glutamine + inosine aerobic		3.29	0.32	0.56	4.18
+ rotenone	7.30	< 0.24	0.49	1.82	2.31
Glutamine + inosine + glucose aerobic		2.87	0.67	0.59	4.13
+ rotenone	6.65	1.99	0.32	0.90	3.22

significantly higher than in any other rotenone-treated cells. This means that, in the presence of glutamine plus inosine, the energy of glycolysis can partially maintain the pool of adenine nucleotides.

It is important to note that there was a significant decrease in pH of the incubation medium if the cells were incubated with glucose, in spite of the high buffering capacity of the medium. However, pH changes of the incubation medium in the range 6.5–7.4 had no effect on the increase in the pool of adenine nucleotides in the presence of glutamine plus inosine (results not shown).

Effect of the concentration of P_i on the total pool of adenylates

The experiments carried out with EAC cells show that an increase in the concentration of extracellular P_i had a significant influence on the effect of glutamine plus inosine on the pool of adenine nucleotides, and that this effect was absent in the control (no addition of glutamine plus inosine) (Table 2). Extension of cell incubation under anaerobic conditions and in the presence of glutamine plus inosine resulted in a marked decrease in the total pool in spite of the large difference in the concentration of phosphate, which is a strong inhibitor of AMP deaminase.

Effect of hadacidin on the level of aspartate in EAC cells under aerobic conditions

Initially the cells were incubated aerobically in the presence of glutamine to increase the intracellular concentration of aspartate, and then washed with the incubation medium to remove the remaining glutamine. The incubation was continued in the presence of inosine and hadacidin for the next 80 min. These experiments represent a direct extension of recently published work [12]. It was expected that there would be no effect of hadacidin on utilization of aspartate if the cells were permanently incubated in the presence of oxygen so that the pool of adenine nucleotides and the concentration of adenylates remain constant. The results presented in Table 3 support this assumption. There is a decrease in the concentration of aspartate (plus glutamate) with time, but there is no significant difference between the controls and cells incubated in the presence of hadacidin. This means that the observed decrease in aspartate is probably due to its oxidation, but not due to its utilization in the resynthesis of adenine nucleotides. Also, there was an increase in ATP in the controls, but not in the presence of hadacidin.

Regeneration of the pool of adenine nucleotides in AS 30D hepatoma cells

Since it was found that AS 30D hepatoma cells have very low synthesis of aspartate from glutamine [14], the question arose of how they regenerate the pool of adenine nucleotides after anoxia. Unlike EAC cells, hepatoma cells do not increase their total pool

Table 2. Effect of extracellular concentration of P_i on the pool of adenine nucleotides (AdN) in EAC cells

The cells were prepared in Krebs–Ringer phosphate medium without phosphate but with 40 mM-Mops/Tris. Additions: phosphate as indicated, 6.3 mM-glutamine, 0.95 mM-inosine and 153 mg wet wt. of cells/ml. All measurements are expressed in nmol/mg wet wt. of cells

$[P_i]$ (mM)	Aerobic (40 min) (control)				Aerobic (40 min) (glutamine + inosine)				Anaerobic (30 min) (glutamine + inosine)			
	ATP	ADP	AMP	AdN	ATP	ADP	AMP	AdN	ATP	ADP	AMP	AdN
0	1.73	0.24	0.42	2.39	1.89	0.24	0.42	2.54	< 0.24	0.36	1.27	1.63
7	1.76	0.23	0.38	2.38	2.20	0.30	0.36	2.86	< 0.24	0.30	1.22	1.52
14	1.72	0.19	0.41	2.32	2.39	0.26	0.48	3.13	< 0.24	0.30	1.24	1.54
29	1.72	0.21	0.38	2.32	2.81	0.29	0.49	3.53	< 0.24	0.35	1.36	1.71
48	1.54	0.24	0.43	2.27	3.36	0.31	0.56	4.25	< 0.24	0.35	1.49	1.84

Table 3. Effect of hadacidin on the levels of ATP, aspartate and glutamate in EAC cells incubated under aerobic conditions

The cells loaded with aspartate were incubated in Krebs-Ringer phosphate solution, pH 7.4. Additions: 4 mM-glutamine, 1.3 mM-inosine, 167 µg of hadacidin/ml. The cell suspension contained 110 mg wet wt. of cells/ml. All measurements are expressed nmol/mg wet wt. of cells.

Time (min)	Control				+ hadacidin			
	ATP	Asp	Glu	Asp+Glu	ATP	Asp	Glu	Asp+Glu
0	2.87	9.26	6.45	15.71	2.57	9.17	5.91	15.08
20	2.96	8.05	7.38	15.43	2.38	7.56	7.30	14.86
40	3.59	6.20	7.08	13.28	2.20	7.58	5.76	13.34
60	3.47	6.97	6.99	13.96	2.17	7.88	6.61	14.49
80	3.10	6.12	5.65	11.77	2.42	7.57	3.98	11.55

of adenylates in the presence of glutamine and inosine. Further investigation revealed that for the resynthesis of adenine nucleotides hepatoma cells need adenosine instead. This is demonstrated by the experiments presented in Fig. 1. In order to decrease the pool of adenylates, the cells were incubated anaerobically (under N₂) for 120 min. However, the total pool did not change very much, which means that, as in EAC cells, we are dealing with the stable pool of adenylates [12]. This was followed by cell incubation for the next 25 and 50 min aerobically in the presence of glutamine plus inosine, with adenosine or with adenosine plus hadacidin. Glutamine plus inosine only slightly increased resynthesis of adenylates, but the effect of adenosine was very significant; 1 mM-inosine had no effect, whereas 0.25 mM-adenosine was enough to obtain almost a full effect on the pool (results not shown). The inhibitory effect of hadacidin was much less pronounced than the corresponding effect on EAC cells, suggesting that resynthesis of adenine nucleotides in hepatoma cells proceeds probably via adenosine kinase rather than via the purine nucleotide cycle.

Kinetics of degradation and resynthesis of the pool of adenine nucleotides in EAC and AS 30D hepatoma cells

Fig. 2 shows the time course of degradation of the pool of

Fig. 1. Regeneration of the adenine nucleotide pool in AS 30D hepatoma cells after long anoxia

After taking samples for the measurement of adenylates, the cells were incubated for 120 min in an atmosphere of N₂. The incubation was continued for the next 50 min aerobically in the presence of glutamine + inosine (G + I), adenosine (A), adenosine + hadacidin (A + H) or without any addition (C). □, ATP; ■, total adenylates (AdN).

adenine nucleotides in EAC and hepatoma cells. The cells were incubated for 30 min with glutamine plus inosine (EAC) or with adenosine (hepatoma) in order to increase the total pool of adenylates. After samples were taken for measurements of the nucleotides, rotenone was added and incubation continued for the next 15 min. Kinetic analysis revealed that there is a markedly faster decrease in ATP concentration in EAC cells than in the hepatoma, so that after about 15 min ATP virtually disappears, whereas hepatoma cells keep their ATP much longer. After 60 min there is still approx. 1 mM-ATP in the hepatoma cells. Similar effects were observed if hepatoma cells were incubated in the absence of oxygen instead of with rotenone. However,

Fig. 2. Kinetics of degradation of the pool of adenine nucleotides in EAC and AS 30D hepatoma cells in the presence of rotenone

To increase the level of the pool, the cells were incubated aerobically for 30 min in the presence of 5 mM-glutamine plus 1 mM-inosine (EAC) or 1 mM-adenosine (hepatoma) before addition of rotenone. O, ATP; ●, ADP; △, AMP; ▲, total pool.

Fig. 3. Kinetics of resynthesis of the pool of adenine nucleotides in EAC and AS 30D hepatoma cells

The cells were incubated anaerobically for 120 min under N_2 . In EAC cells the incubation was continued aerobically in the presence of 5.8 mM-glutamine plus 1 mM-inosine, whereas AS 30D hepatoma cells were incubated under same conditions but in the presence of 1 mM-adenosine. The cell suspensions contained 158 and 152 mg wet wt. of cells/ml respectively. $\circ$, ATP; $\bullet$, ADP; $\triangle$, AMP; $\blacktriangle$, total pool.

addition of 0.1 mM-iodoacetate resulted in a very rapid disappearance of ATP under anaerobic conditions, which is in accordance with data reported by Plagemann & Wohlhuetter [16]. It should be pointed out that there was a rapid decrease in the ATP/ADP ratio, in spite of concomitant deamination of AMP and displacement of the reaction of adenylate kinase to the direction of ATP synthesis.

Analysis of the rate of adenine nucleotide resynthesis shows that there is a very rapid regeneration of ATP and the total pool of adenylates after reoxygenation of the cell suspension (Fig. 3). Before reoxygenation the cells were incubated for 120 min under N_2 . Regeneration of the pool was especially rapid in hepatoma cells.

DISCUSSION

Experimental data reported in this paper revealed several features of the kinetics and mechanism of degradation and resynthesis of the pool of adenylates in the tumour cells studied. The results presented in Table 1 show that, although glucose is an important substrate for tumour energy metabolism, glutamine (plus O_2) is absolutely necessary for the maintenance of a high level of the adenine nucleotide pool in EAC cells. It was reported that during exponential growth of cultured mammalian cells the total adenylates ranged from 10 to 11 fmol/cell, whereas with increasing cell density and declining growth total adenylates decreased to a minimum of 4.5 fmol/cell [17]. It is significant that the change in total adenine nucleotides precedes alteration in DNA synthesis and mitosis [18]. It is possible that the same happens in malignant cells, especially if they are in the ascitic form. In spite of their ability to live under prolonged anaerobic conditions, there is no DNA replication and cell division without oxygen. This could be related to the level of total adenylates, but also to the requirement of oxygen for synthesis of pyrimidines *de novo* [19].

In accordance with our previous experimental data, cell incubation with glutamine plus inosine markedly increased the level of ATP and total adenylates [12]. Addition of rotenone in this case decreased the concentration of ATP below the limit of detectability, whereas the total pool of adenylates was diminished to approx. 45%. If in addition to these two metabolites glucose were present, rotenone did not decrease the concentration of

ATP below 2.8 mM, and the total pool was not decreased by more than 22%. This can be explained by the fact that, in addition to the components needed for AMP regeneration (aspartate plus inosine), the formation of ATP and GTP occurs in the presence of glucose, and this is important for the reaction of adenylosuccinate synthetase [20].

Further investigation of the factors which have influence on the effect of glutamine on the level of adenylates in EAC cells showed that the effect depends on the concentration of P_i . Increasing the concentration of extracellular P_i from 0 to 48 mM had a significant effect on the increase in the pool of adenine nucleotides with glutamine plus inosine. The mechanism of this increase in the pool of adenylates by P_i is not clear. It could be speculated that this is due to activation of glutaminase, which is a phosphate-dependent enzyme. It is possible, however, that P_i increases synthesis of 5-phosphoribosyl 1-pyrophosphate, since phosphoribosyl pyrophosphate synthetase (ribose-5-phosphate pyrophosphokinase, EC 2.7.6.1) has an absolute requirement for P_i as an activator. The very high concentration of phosphate did not prevent a decrease in the total pool of adenylates under anaerobic conditions, although phosphate is a strong inhibitor of AMP deaminase (results not shown). This throws some doubt on the role of this enzyme in degradation of the pool of adenylates. However, analysis of cell extracts by h.p.l.c. showed clearly that degradation of AMP is followed by accumulation of IMP, indicating that AMP deaminase was activated (results not shown).

It is known that the purine nucleotide cycle is not the only mechanism for regeneration of the adenine nucleotide pool or resynthesis of AMP. There are other possibilities, such as the reaction of adenosine kinase, which is important for the heart and probably for liver [21–23]. Therefore, two completely different enzymic systems could have the same function in the degradation and resynthesis of adenylates. These are the purine nucleotide cycle and the system 5'-nucleotidase (soluble) adenosine kinase. According to Atkinson [5], both pathways could have the same effect or function: the maintenance of a normal ATP/ADP ratio. This functional approach may be very important for understanding tumour biochemistry, and especially the heterogeneity of the tumour phenotype. Our investigation carried out with AS 30D hepatoma cells showed that the predominant mechanism for AMP regeneration is probably the

reaction of adenosine kinase (Fig. 1). For that reason, addition of hadacidin had a very small effect, corresponding to the part of resynthesis for which the activity of the purine nucleotide cycle is responsible. It is interesting that these cells have a very low rate of glutamine consumption and almost insignificant production of aspartate and CO_2 [14]. This could be simply a coincidence, but it strongly suggests that an important function of a high rate of glutamine oxidation via the aspartate aminotransferase pathway in some rapidly dividing tumour cells, e.g. EAC cells, is resynthesis of adenine nucleotides after aerobic-anaerobic transitions, as proposed elsewhere [12].

In addition to other mechanisms of adaption, tumour cells develop an efficient way for a rapid regeneration of the pool of adenine nucleotides after the end of ischaemia. A previous investigation in this field revealed that regeneration of adenine nucleotides in normal cells is a slow process [21]. The data obtained with EAC and AS 30D hepatoma cells in the present study showed that resynthesis of the pool is complete in about 20 min on reoxygenation after 2 h of anaerobiosis if glutamine plus inosine or adenosine were added to the incubation medium (Fig. 3).

Although operation of the purine nucleotide cycle was demonstrated in both normal [24,25] and malignant tissues [12], its metabolic function is still an open question. The problem is what causes the activation of AMP deaminase and rapid degradation of only part of the total pool of adenylates, which we call the variable pool [12]. Lowenstein and co-workers [26] pointed out that the reactions of the purine nucleotide cycle are dissected into phases corresponding to 'energy drain' and 'energy excess', which are reflected by AMP hydrolysis and resynthesis respectively. It was proposed that an important function of the cycle is to maintain a high ATP/ADP ratio by activating AMP deaminase and coupling this reaction with the reaction of adenylate kinase. In support of this, a correlation between adenylate energy charge and activity of AMP deaminase was found [27]. However, kinetic analysis of degradation of adenylates throws some doubt on the assumption that the purine nucleotide cycle is an energy buffer in these cells. There is a rapid decrease in the ATP/ADP ratio in both kinds of tumour cells studied, but the questions still remains how much of ADP is protein-bound and, therefore, has no influence on the ratio.

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