Interaction of metabolism of aspartate and inosine and energy state of malignant cells

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1. Oxidation of glutamine in Ehrlich ascites-carcinoma cells results in a large accumulation of aspartate.
2. The addition of inosine causes a marked decrease in aspartate production from glutamine. This may be related to the resynthesis of AMP from aspartate and IMP, the latter being produced from inosine via the salvage pathway for purine nucleotides. In accordance with this assumption, a significant production of lactate was observed, which comes probably from the ribose moiety of inosine. Since lactate is known to inhibit production of aspartate from glutamine, this may explain the effect of inosine.
3. Addition of glutamine together with inosine increased cellular ATP content. This was not the case if glutamine or inosine was present separately or if inosine was added together with lactate, pyruvate or glucose. The effect did not occur if amino-oxyacetate, an inhibitor of transaminases, was added. These findings suggested again that production of aspartate is important for resynthesis of ATP from IMP via the purine nucleotide cycle.
4. If the cells were exposed to prolonged anaerobic incubation, addition of glutamine and inosine markedly increased O_2 uptake and [ATP], suggesting the crucial importance of aspartate production by glutamine oxidation for the recovery of energy metabolism in the cells.

INTRODUCTION

Apart from the biosynthetic aspect of glutamine metabolism, which is very important for malignant cells, especially for synthesis of purines and pyrimidines *de novo* [1], there is another aspect important for the study of glutamine utilization, concerning its oxidation and its role in energy metabolism of tumours. It was reported from several laboratories that glutamine is an important energy fuel for cancer cells ([2]; for reviews see [3–5]). However, the reason for the high rate of glutamine oxidation in rapidly dividing cells is still unclear. It has been emphasized that the oxidation is only partial, and is important for providing precursors for biosynthetic reactions rather than for energy metabolism [6]. In relation to this problem, our interest was focused on aspartate as one of the most important intermediates in glutamine oxidation. The question arose whether aspartate could be an important metabolite for tumour cell energy metabolism. We previously reported that the activity of the malate–aspartate shuttle is dependent, at least in some tumour cell lines, on the concentration of aspartate [7]. Another pathway important for energy metabolism, which might impose a large demand for aspartate in tumours, is the purine nucleotide cycle. It has been demonstrated by Lowenstein and his collaborators [8] that the cycle is very important for energy metabolism in skeletal muscle. Weber and co-workers found that there is a transformation-linked increase in activities of adenylosuccinate synthase (EC 6.3.4.4) and adenylosuccinate lyase (EC 4.3.2.2) in hepatomas, whereas the activity of AMP deaminase (EC 3.5.4.6) is increased in correlation with tumour growth rate [9]. This can be explained in terms of a great demand for IMP, which is the key metabolite for purine nucleotide synthesis. However, there is also another possibility which might be equally important for tumour cells. This is related to the regulation of adenylate energy charge and the resynthesis of AMP after sudden metabolic stress

such as anoxia or ischaemia, which occurs frequently in malignant tissue *in vivo* (for review see [10]). During ischaemia there is a rapid decrease in [ATP], followed by an increase in [AMP]. Activation of AMP deaminase under these conditions will lead to a decrease in [AMP]. This is important for cellular energy metabolism, because activation of this enzyme in co-operation with the adenylate kinase reaction increases the ATP/ADP ratio. This ratio is even more important for cellular energy metabolism than is the concentration of ATP [11]. After the end of the ischaemic period, the pool of adenine nucleotides must be regenerated through the reactions of adenylosuccinate synthase and adenylosuccinate lyase which result in net utilization of aspartate. Accordingly, it is proposed that aspartate produced during oxidation of glutamine plays an important role in the frequent demand of malignant cells for regeneration of the adenine nucleotide pool and adaptation of tumour energy metabolism to the transition of cells from anaerobic to aerobic conditions. This could be of particular significance for the process of tumour progression.

MATERIALS AND METHODS

All the experiments were performed *in vitro* with Ehrlich ascites-carcinoma cells. The cells were propagated in the abdominal cavity of Swiss mice and harvested usually 7 days after transplantation. They were washed twice in Krebs–Ringer phosphate solution of the following composition: 145 mM-NaCl, 6 mM-KCl, 1.5 mM- KH_2PO_4 , 15 mM- Na_2HPO_4 , 1.5 mM- $MgSO_4$. The final pH was 7.4 and the temperature was 0 °C. 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. This was important, especially if the cells were incubated with glucose, since they possess a large capacity for lactate production. Incubation of the cells was carried

Fig. 1. Production of aspartate from glutamine, asparagine and proline

The cells were incubated in the presence of 6 mM-glutamine (●), 6 mM-asparagine (○) and 6 mM-proline (△).

out in 25 ml Erlenmeyer flasks under air. The layer of the cell suspension (approx. 120–130 mg wet wt. of cells/ml) was thin enough to avoid lack of O_2 . All incubations, except when O_2 uptake was measured, were performed in a Dubnoff metabolic shaker. After the incubation, the reaction was terminated by addition of $HClO_4$ (final concn. 10%, w/v). The cell extract was neutralized with 5 mM-KOH, and $KClO_4$ was precipitated by centrifugation (2.5 min at 15000 g).

Concentrations of metabolites (aspartate, lactate, ammonia, AMP and ATP) were determined by standard enzymic methods [12]. Cell respiration was measured by using a Clark oxygen electrode. All measurements were done in triplicate, and the means of three determinations were shown. The crucial experiments (Table 2 and Fig. 4) were repeated three or four times and the results shown are representative of a reproducible effect. All measurements are expressed per mg wet wt. of cells

RESULTS AND DISCUSSION

Production of aspartate from glutamine, asparagine and proline

Fig. 1 shows that glutamine represents the most important source of aspartic acid for the ascites-tumour cells. The cells take up glutamine rapidly, and after its hydrolysis glutamate (plus NH_4^+) is formed, which is oxidized mainly through the transamination pathway (for reviews see [3] and [4]). Another possible source of aspartate from the extracellular medium *in vivo* could be glutamate, proline and asparagine. However, the plasma membrane of the cells is virtually impermeable for glutamate [13], as for other dicarboxylic acids. Proline could be an important source of aspartate, since there is the possibility for its transformation into glutamate, and it is a good substrate for respiration of isolated mitochondria from different sources and for the synthesis of aspartate in some normal cells (hepatocytes, kidney tubule cells) [14,15]. However, our investigation showed that proline, and also asparagine, compared with glutamine are very poor sources of aspartate for ascites-tumour cells (Fig. 1).

It should be pointed out that the synthesis of aspartate from glutamine proceeds linearly for at least 2 h, reaching a very high intracellular concentration of approx. 20 mM.

Fig. 2. Inhibition of aspartate production by inosine

The cells were incubated in the presence of 8 mM-glutamine without (○) or with (●) 5 mM-inosine.

This indicates that utilization of aspartate is slow compared with the rate of its synthesis. The endogenous concentration of aspartate measured immediately after harvesting cells from the ascites fluid was 0.5–1.0 mM. Glucose, lactate, pyruvate and palmitoylcarnitine, sources of acetyl-CoA, cause a strong inhibition of aspartate production (results not shown) [7,16]. This can be explained by the competition of citrate synthase (EC 4.1.3.7) and aspartate aminotransferase (EC 2.6.1.1) for oxaloacetate. It is possible, however, that *in vivo* the concentration of aspartate is limited by hypoxia or by a low concentration of glutamine in the ascitic fluid. Nevertheless, these cells have a large capacity for the production and accumulation of aspartate.

Effect of inosine on the production of aspartate

If the cells were incubated with glutamine plus inosine, there was a significant decrease in production of aspartate (Fig. 2). The effect was not observed if adenosine was present instead of inosine. These findings indicate that there is a strong interaction between metabolism of inosine and aspartate.

Production of lactate from inosine

It was assumed that the mechanism of the decrease in aspartate production in the presence of inosine might be its utilization in the purine nucleotide cycle. In this case one might expect an increase in AMP, but we were not able to demonstrate this (Table 1). The next assumption was that there is an increase in AMP production,

Table 1. Effect of inosine (5 mM) and glutamine (6 mM) on the content of AMP and production of ammonia in Ehrlich ascites-carcinoma cells

All the measurements were done in triplicate.

Time (min)	AMP (nmol/mg)		NH_3 (nmol/mg)	
	Inosine	Glutamine + inosine	Glutamine	Glutamine + inosine
0	0.19	0.19		
30	0.33	0.19	23.7	24.2
60	0.31	0.19	34.2	36.8
90	0.43	0.26	49.3	44.7
120	0.48	0.28	52.6	57.9

Fig. 3. Production of lactate from inosine, and the effect of glutamine and arsenite

(a) The amount of lactate produced was measured in cells which were incubated in the presence of inosine (Δ), inosine plus glutamine ($\bullet$), or glutamine ($\blacktriangle$); control ($\circ$). (b) The net production of lactate was measured in cells which were incubated with inosine ($\circ$), inosine plus arsenite ($\bullet$) and glutamine plus arsenite (Δ). The added compounds were present at the following concentrations: 5 mM-inosine, 8 mM-glutamine and 1 mM-arsenite.

followed by rapid deamination in the AMP deaminase reaction. In that case an increase in ammonia production might be expected, but this assumption was not supported by experimental findings, either (Table 1). This suggests that activity of the purine nucleotide cycle in the cells is probably low. (It should be pointed out that there is a difficulty in measuring production of ammonia from AMP in these experiments, because of its large production from glutamine.) Therefore we were searching for another way to explain the effect of inosine. If there is utilization of aspartate in the purine nucleotide cycle, one might expect production of fumarate and malate. According to Moreadith & Lehninger [17], the mitochondria of Ehrlich ascites-carcinoma cells metabolize extra-mitochondrial malate in the reaction of NAD(P)-dependent malic enzyme (EC 1.1.1.39) resulting in the production of CO_2 and pyruvate. The question arose whether the same happens in the present experiments as well. This interpretation is supported by the finding by Sauer *et al.* [18] that the mitochondria of malignant cells contain an active malic enzyme, the function of which could be net oxidation of glutamine, as well as reduction of NAD(P)^+ . Experimental data of Watford *et al.* [19]

indicate that, for the net oxidation of glutamine in rat kidney, the activity of malic enzyme is important too, and that this leads to the production of lactate. Production of lactate from glutamine was found in fibroblasts in tissue culture and in enterocytes [20,21]. Our experiments, in which ascites cells were incubated with glutamine plus inosine, revealed that the cells produce a considerable amount of lactate (Fig. 3a). However, further investigation showed that lactate is formed from inosine itself, probably from its ribose moiety, which is then metabolized via the pentose phosphate pathway [10]. Although the addition of glutamine stimulates production of lactate from inosine, the cells did not synthesize lactate from glutamine, indicating that they do not possess an appreciable activity of malic enzyme. Stimulation of lactate formation from inosine by glutamine results probably from the competition between glutamine and pyruvate for oxidation in the tricarboxylic acid cycle. This interpretation is supported by experiments with arsenite, which increases accumulation of lactate from inosine, owing to inhibition of pyruvate oxidation (Fig. 3b).

According to the data of Moreadith & Lehninger [17], one might also expect production of alanine rather than lactate from glutamine via mitochondrial malic enzyme. Although this could occur in isolated mitochondria, it is probably less likely in the cells, because they possess enormous activity of lactate dehydrogenase, which channels pyruvate into lactate (unless efflux of pyruvate from the mitochondria is very slow). In our previous experiments with isolated Ehrlich ascites-tumour-cell mitochondria, we could not demonstrate production of alanine from glutamine even in the presence of pyruvate [16]. If there is significant production of alanine in the tumour cell line, one would expect a decrease rather than an increase in lactate production from inosine in the presence of glutamine. Also, the large production of aspartate in the cells studied strongly supports the conclusion that the aspartate aminotransferase rather than the alanine aminotransferase (EC 2.6.1.2) pathway predominates and that there is no significant production of pyruvate via malic enzyme, since pyruvate would inhibit aspartate formation [7,16].

ATP content in cells incubated with glutamine and inosine

Measurement of the ATP content during incubation of the cells with glutamine and inosine revealed that the content of this high-energy phosphate compound is increased more than 2-fold, leading to a profoundly different energy state of the cells (Fig. 4a). If the cells were incubated with glutamine or inosine separately, the effect was absent. Incubation of the cells with inosine plus glucose increased ATP, but not as much as with inosine plus glutamine (Fig. 4b). Even the addition of glutamine under these conditions did not make a significant difference. A similar observation was made if the cells were incubated with inosine plus pyruvate or inosine plus lactate (Fig. 4d). It should be pointed out that all three metabolites (glucose, lactate and pyruvate) decrease the production of aspartate. Fig. 4(c) shows that the addition of amino-oxyacetate, which is an inhibitor of aspartate aminotransferase, abolishes the effect of glutamine and inosine on the ATP content.

The same effect, but even more pronounced, of glutamine and inosine on ATP content was observed if

Fig. 4. Effect of glutamine and inosine on the content of ATP in ascites-carcinoma cells

(a) Effect of glutamine plus inosine (●), glutamine (△), inosine (▲); control (○). (b) Effect of glucose plus inosine (▲), glucose, inosine and glutamine (△), inosine plus glutamine (●); control (○). (c) Effect of amino-oxyacetate, inosine and glutamine (▲), inosine plus glutamine (●), amino-oxyacetate (△); control (○). (d) Effect of lactate plus inosine (△), pyruvate plus inosine (▲), glutamine plus inosine (●); control (○). Concentrations of metabolites and of the inhibitor were as follows: 8 mM-glutamine, 5 mM-inosine, 10 mM-glucose, 10 mM-lactate, 8 mM-pyruvate and 1 mM-amino-oxyacetate.

the cells were damaged by prolonged incubation under anaerobic conditions. Besides severe depletion of ATP, the cells showed a very low rate of respiration, which was markedly increased if they were incubated with glutamine and inosine before measurement of O_2 uptake (Table 2). There are several possible explanations for this phenomenon: (1) accumulation of substrates which serve as energy fuels for the cells; (2) accumulation of aspartate and stimulation of the malate-aspartate shuttle; (3) increase of the pool of adenine nucleotides, through accumulation of aspartate as a precursor for the synthesis of AMP. The first possibility is partially true, because incubation of the cells with glucose or lactate stimulates respiration as well, but the stimulation is significantly less than with glutamine. The possibility of stimulation of the malate/aspartate shuttle was the subject of a previous investigation [7].

The finding that glutamine and inosine markedly increase ATP content and the rate of respiration, thereby improving the energy state of the cells, is of particular importance. The addition of some other substrates, such as glucose, lactate or pyruvate, does not have so pronounced an effect as the addition of glutamine. It is reasonable to assume that the adenine nucleotide pool of the cells is depleted and that the addition of inosine replenishes IMP through the salvage pathway of purine nucleotides, whereas the addition of glutamine provides aspartate. Therefore, both key metabolites needed for the

Table 2. Oxygen uptake by ascites-carcinoma cells and the ATP content

The cells were preincubated for 80 min under N_2 and then incubated for 40 min aerobically in the presence of different substrates. After centrifugation, the supernatant was removed and the pellet was resuspended in the incubation medium. The cell suspension was used for the measurement of O_2 uptake. ATP was measured after termination of aerobic incubation. The measurements were done in triplicate. Concentrations of the substrates: 8 mM-glutamine, 5 mM-inosine and 10 mM-glucose.

Addition of substrates	O_2 uptake (ng-atoms/min per mg)	ATP (nmol/mg)
Control	0.35	0.1
Glutamine + inosine	1.80	1.9
Glucose + inosine	0.92	0.5
Glutamine + glucose	0.82	0.6
Stock cell suspension	1.32	1.1

synthesis of AMP become available. The fact that there is no accumulation of AMP or production of ammonia suggests that AMP is channelled into the direction of ATP synthesis. This means that inosine and glutamine,

or aspartate, are very important for recovery of cellular energy metabolism and for repair of reversible damage, especially to membranes, which occurs during hypoxia or ischaemia. This is in accordance with observations by Bush *et al.* [22], who showed that the addition of aspartate and glutamate to the physiological solution used in cardiopulmonary bypass surgery can protect the ischaemic myocardium. Krebs & Lund [23] pointed out that glutamine, glutamate and aspartate can act as 'sparkers' of the tricarboxylic acid cycle. Their explanation was that sparkers supply oxaloacetate, which promotes the citrate synthase reaction and thereby regenerates CoA, which may be the rate-limiting factor for fatty acid removal. They concluded, however, that there are certain sparker effects which cannot be explained on this basis. Our experimental data suggest that some of these effects are probably related to the resynthesis of AMP and ATP.

We thank Professor G. Weber for helpful discussions throughout this work and for critical reading of the manuscript. This material is based on work supported by the U.S.-Yugoslav Joint Fund for Scientific and Technological Cooperation, in co-operation with National Institutes of Health, U.S.A., Grant No. 699.

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Received 15 January 1987/19 May 1987; accepted 11 June 1987