The role of glutamine oxidation and the purine nucleotide cycle for adaptation of tumour energetics to the transition from the anaerobic to the aerobic state

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It is proposed that the purine nucleotide cycle and glutamine oxidation play a key role in the adaptation of tumour energetics to the transition from the anaerobic to the aerobic state. In support of this proposal, it was found that glutamine and inosine markedly increase total adenylates in the presence of oxygen, whereas the addition of hadacidin abolishes this effect. Transition of the cells from the anaerobic to the 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 transition. This part of the pool undergoes degradation or resynthesis owing to activation of the enzymes of the purine nucleotide cycle. Resynthesis of the pool is accompanied by substantial net utilization of aspartate, which is produced by glutamine oxidation. This is supported by the experiments in which the cells were alternately incubated with nitrogen or oxygen, demonstrating that hadacidin significantly decreased utilization of aspartate and regeneration of ATP owing to inhibition of adenylosuccinate synthase.

INTRODUCTION

Irrespective of whether damage to cell respiration causes malignant transformation, there is no doubt that tumour progression is accompanied by a profound change in energy metabolism [1]. One of the main reasons is the fact that tumour growth is never followed by an adequate vascularization, so that the cells are faced with episodes of shorter or longer hypoxia or ischaemia, to which they have to adapt [2]. Besides the well-known increase in glycolysis, one of the mechanisms of this adaptation is utilization of some metabolites as energy fuel, although they are not normally used for this purpose. In relation to this, our investigation of Morris hepatomas revealed that glutamine is a very important energy fuel for these tumours [3]. This observation was confirmed in several laboratories with several different tumour cell lines, but the reason for the high rate of glutamine oxidation in rapidly dividing cells remained without satisfactory explanation (for reviews, see [4] and [5]). Further investigation showed that the oxidation of glutamine results in a large production of aspartate, which is important, as far as energy metabolism is concerned, for the operation of the malate–aspartate shuttle [6]. However, aspartate plays a catalytic role in the shuttle, and there is no need for its net utilization. The most recent explanation was proposed by Coleman and collaborators [7], who observed that a high and uncontrolled cholesterol synthesis in tumours is supported by a rapid efflux of citrate from the mitochondria. This causes inability of tumour mitochondria to oxidize citrate via the tricarboxylic acid cycle. For this reason only a small percentage of pyruvate produced from glucose is oxidized. An alternative substrate which can replace citrate is glutamine, which is directly connected with the tricarboxylic acid cycle via glutamate and 2-oxoglutarate. However, recent experimental and especially theoretical work by Newsholme and collaborators throws serious doubt on the proposal concerning the importance of glutamine oxidation for tumour-cell energetics [8]. They emphasized that this oxidation is only partial and important for providing precursors for biosynthetic reactions. This renewed our interest in the problem, and focused our attention on aspartate as one of the most abundant intermediates of glutamine oxidation. An important pathway of energy metabolism which might have a large demand for aspartate, especially in tumours, is the purine nucleotide cycle. It was shown by Lowenstein and co-workers [9] that this cycle plays an important role in skeletal-muscle energetics during transition from the aerobic to the anaerobic state, and vice versa. The work of Weber and his collaborators [10] clearly showed that all the enzymes of the purine nucleotide cycle have an increased activity in malignant tumours so far examined.

Our main proposal was that transition of tumour cells from the aerobic to the anaerobic state, and vice versa, is accompanied by a change in the pool of adenine nucleotides, and that the production of aspartate is very important for regeneration of the pool during the aerobic phase. This was supported by some recently reported experimental findings [11], but in the present paper we provide more direct evidence for this proposal.

MATERIALS AND METHODS

Ehrlich ascites-carcinoma cells were used throughout this study. All experiments with the cells were carried out *in vitro*. The details of experimental conditions, propagation and preparation of the cells were described elsewhere [11] and in the Figure legends. The cells were washed twice in Krebs–Ringer phosphate buffer of the following composition: 145 mM-NaCl, 6 mM-KCl, 1.5 mM-KH_2PO_4, 15 mM-Na_2HPO_4 and 1.5 mM-$MgSO_4$.

The final pH was 7.4 and the temperature was 0 °C. The incubation medium was of the same composition, except that the concentration of Na_2HPO_4 was 7.5 mM. Also, buffer capacity of the medium was increased by the addition of 20 mM-Tris/HCl and 20 mM-Mops. The final pH was also 7.4 and the temperature was 30 °C. Incubation of the cells was carried out in 25 ml Erlenmeyer flasks under O_2/CO_2 (19:1) or under N_2. The cell suspension contained approx. 130 mg wet wt. of cells/ml, and the layer of suspension was thin enough to avoid lack of O_2. All incubations, except when O_2 was measured, were done in a Dubnoff metabolic shaker. The details of experimental protocol are described in the Figure legends. Incubation was terminated by addition of cell suspension to cold $HClO_4$ (final concn. 10%, w/v). The cell extract was neutralized with 5 M-KOH in the presence of Methyl Orange, and $KClO_4$ was precipitated by centrifugation, in an Eppendorf centrifuge.

Concentration of metabolites (ATP, ADP, AMP, aspartate and glutamate) were determined by standard enzymic methods [12]. Recovery of AMP and ADP was 90%, and that of ATP was 80%. It was assumed that approx 20% of the cell pellet belongs to the extracellular space. This was taken into account in the calculation of intracellular concentrations of the metabolites. All the experiments were repeated two to four times with different batches of the cells, and representative results are presented in the Figures. The measurements of the metabolites were done in triplicate, and means of three determinations are recorded.

Cell respiration was measured by a Clark oxygen electrode.

All results are expressed per mg wet wt. of the cells.

RESULTS

Effect of glutamine, inosine and hadacidin on the pool of adenine nucleotides

We recently reported that addition of glutamine together with inosine markedly increased ATP in ascites-tumour cells [11]. In the experiments presented in Fig. 1, changes in the total pool of adenine nucleotides in the presence of glutamine and inosine and the effect of hadacidin {an inhibitor of adenylosuccinate synthase (EC 6.3.4.4) [13]} were investigated. The cells were incubated for 120 min under N_2 and then N_2 was replaced by O_2 (Fig. 1*a*). In other experiments glutamine and inosine were added together with O_2 (Figs. 1*b* and 1*c*). In the experiments shown in Fig. 1(*c*), hadacidin was added before these metabolites. Measurements of the contents of ATP, ADP and AMP and calculation of the total pool of adenine nucleotides resulted in the following observations: (i) long anaerobiosis did not deplete the adenine nucleotide pool below approx. 1.5 mM; (ii) glutamine and inosine markedly increased the pool in the presence of O_2, whereas the addition of hadacidin abolished this effect.

Effect of glutamine, inosine, rotenone and 2-deoxyglucose on the pool of adenine nucleotides

In these experiments the cells were incubated aerobically for 40 min in the presence of glutamine in order to increase intracellular aspartate. In the experiments presented in Fig. 2(*a*), glutamine was added, whereas in Fig. 2(*b*) glutamine was added together with inosine. This resulted in an increase in the total pool of adenine nucleotides and the content of ATP. Addition of rotenone, which inhibits the respiratory chain, resulted in a decrease in ATP content to undetectable values, together with a concomitant increase in AMP. The total pool of adenine nucleotides did not change significantly (Fig. 2*a*). However, when the cells were incubated with glutamine plus inosine and the adenine nucleotide pool was increased (Fig. 2*b*), the addition of rotenone markedly and rapidly decreased the pool to a value which was similar to the control (Fig. 2*a*). Further incubation did not change the total size of the adenine nucleotide pool. Similar results were obtained if 2-deoxyglucose was added to deplete ATP (Fig. 2*c*). Together with the previous experiments (Fig. 1), this suggests that there are two components of the adenine nucleotide pool. One part of the pool is stable and amounts to approx. 1.5–2.0 mM, whereas the other part is

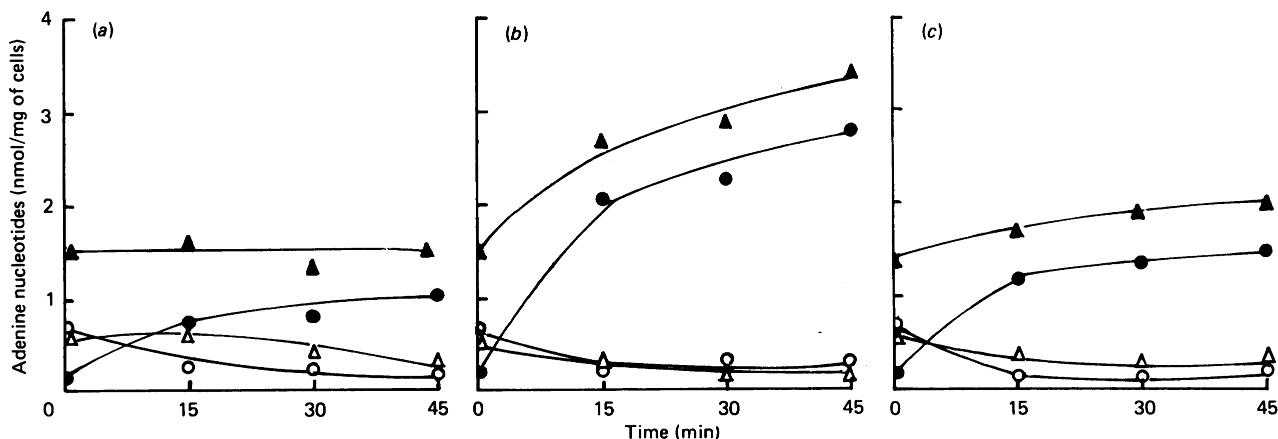


Fig. 1. Effect of glutamine, inosine and hadacidin on the pool of adenine nucleotides

(*a*) The cells were incubated for 120 min under N_2. This was followed by the incubation in the presence of O_2 for the next 45 min. During this time samples were taken for the analysis of adenine nucleotides. (*b*) Same as in (*a*) except that before O_2 5.6 mM-glutamine plus 0.8 mM-inosine were added. (*c*) Same as in (*a*), but before O_2 hadacidin (137 μg/ml), glutamine and inosine were added. ●, ATP; △, ADP; ○, AMP; ▲, AdN (total pool of adenine nucleotides).

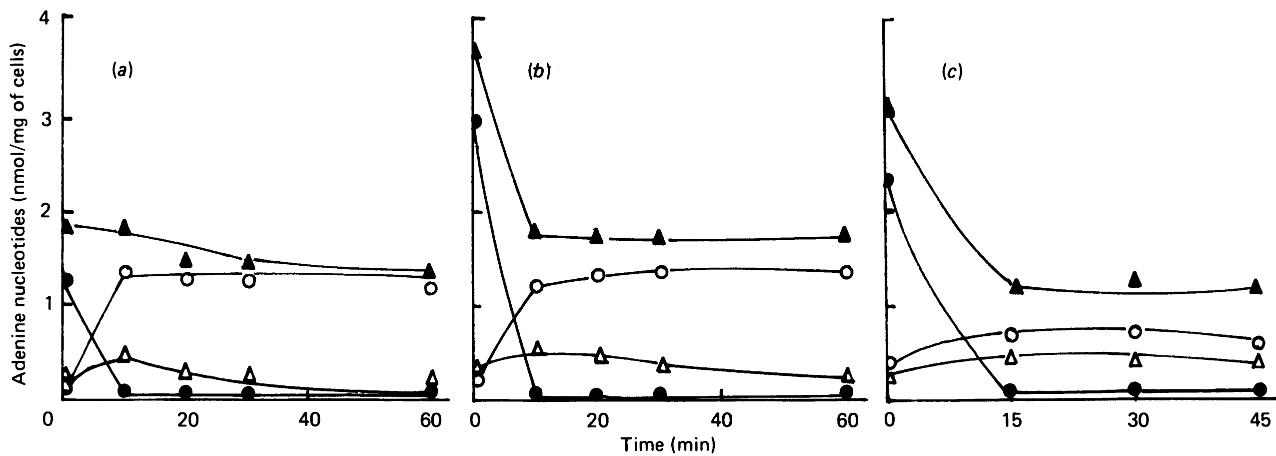


Fig. 2. Effect of inosine, rotenone and 2-deoxyglucose on the pool of adenine nucleotides

(*a*) The cells were incubated aerobically for 40 min in the presence of 4 mM-glutamine. This was followed by addition of rotenone (0.84 μg/ml) and incubation for the next 60 min. ●, ATP; △, ADP; ○, AMP; ▲ AdN. (*b*) Same as in (*a*), except that the cells were incubated with glutamine plus 0.8 mM-inosine before addition of rotenone. (*c*) Same as in (*a*), except that the cells were incubated with glutamine before addition of 5 mM-2-deoxyglucose in place of rotenone.

Table 1. Effect of alternate incubation of the cells with N_2 and O_2 on the content of ATP and concentrations of glutamate and aspartate

In the controls, cells were incubated aerobically for 30 min with 4 mM-glutamine, sedimented by centrifugation, and the supernatant was removed. After resuspension in the medium without glutamine but with the addition of 1 mM-inosine, the cells were alternately incubated for 15 min under N_2 or O_2. The experiment involved five pulses of each gas phase over a total time of 150 min. After the first and the last pulses with N_2 or O_2, samples were taken for measurements of metabolites. In the hadacidin experiment, the conditions were identical, except that 182 μg of hadacidin was added together with 1 mM-inosine. Incubations were with 110 mg wet wt. of cells/ml. The results are expressed in nmol/mg wet wt. of cells.

Anaerobic/aerobic pulses	ATP		Glutamate		Aspartate	
	Control	+Hadacidin	Control	+Hadacidin	Control	+Hadacidin
First cycle						
N_2	< 0.16	< 0.16	4.9	4.9	9.2	9.2
O_2	2.60	1.47	2.8	3.2	7.6	8.1
Last cycle						
N_2	0.66	< 0.16	2.6	3.3	3.7	5.8
O_2	2.00	0.93	2.2	3.2	3.5	5.6

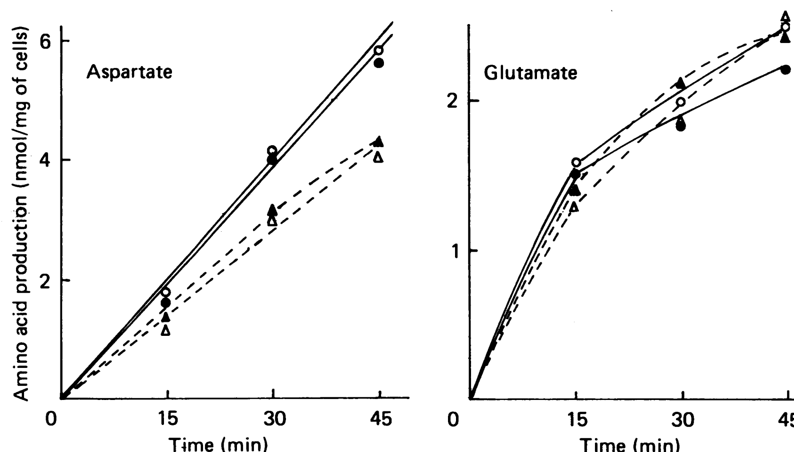
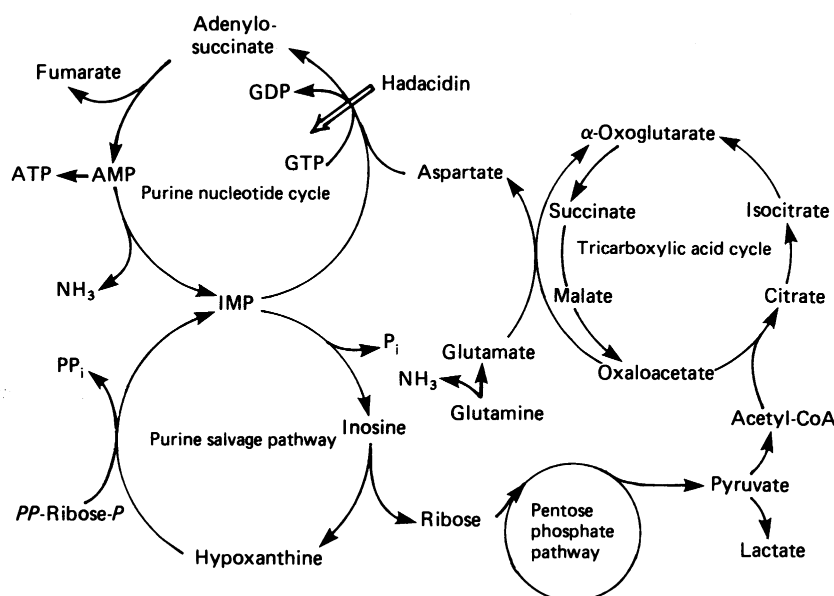


Fig. 3. Effect of hadacidin, inosine and inosine plus hadacidin on aspartate and glutamate production

The cells were incubated aerobically in the presence of 7.7 mM-glutamine without or with the addition of 280 μg of hadacidin/ml, 1.4 mM-inosine or both. ○, Control; ●, hadacidin; △, inosine; ▲, hadacidin plus inosine.



Scheme 1. Metabolic pathways involved in degradation and resynthesis of adenine nucleotides

variable (between 2 and 4 mM) and responds rapidly to the presence or absence of O_2 and glutamine plus inosine. It is proposed that this part of the pool undergoes degradation or resynthesis, owing to activation of the enzymes of the purine nucleotide cycle.

Changes in the content of ATP and concentrations of aspartate and glutamate in cells alternately incubated with N_2 and O_2

The objective of these experiments was to show that the increase in the pool of adenine nucleotides, which is observed after reoxygenation in the presence of glutamine and inosine (variable pool), is accompanied by utilization of aspartate. To achieve a greater depletion of aspartate, which was accumulated by aerobic incubation, the cells were alternately incubated with N_2 and O_2. 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. After the first and the last anaerobic and aerobic pulses, samples were taken for measurements of ATP, aspartate and glutamate (Table 1). These data show that incubation of the cells under N_2 leads to disappearance of ATP, whereas oxygenation leads to its regeneration. During this time (five cycles altogether with N_2 and O_2), there was a decrease in the concentrations of both aspartate and glutamate, which are in equilibrium via the aspartate aminotransferase reaction (Table 1). The same experiment was carried out in the presence of hadacidin which was added before the aerobic–anaerobic cycles. Although there is regeneration of ATP in the presence of hadacidin and O_2, this is markedly less than in the control experiments. The decrease in aspartate and glutamate was significantly smaller in the presence of hadacidin. Hadacidin did not inhibit the utilization of aspartate (plus glutamate) if the cells were incubated continuously for 80 min in the presence of O_2 (results not shown). These findings strongly support our key proposal that regeneration of the adenine nucleotide pool after repeated episodes of hypoxia and ischaemia is accompanied by a substantial utilization of aspartate.

Effects of hadacidin and inosine on the production of aspartate and glutamate

In order to test whether the effects of hadacidin are specifically related to the inhibition of adenylosuccinate synthase, the production of aspartate and glutamate from glutamine was measured in the presence and absence of the inhibitor (Fig. 3). Hadacidin did not inhibit aspartate aminotransferase (EC 2.6.1.1), as judged by the production of aspartate. Similarly, it did not inhibit the production of glutamate (which is in equilibrium with aspartate). Fig. 3 also shows that inosine inhibited the synthesis of aspartate, as reported previously [11], but the addition of hadacidin did not change this effect significantly. This supports our previous conclusion [11] that inhibition of aspartate formation in the presence of inosine and O_2 is mainly due to production of lactate (via the pentose phosphate pathway) and to competition of aspartate aminotransferase and citrate synthase for oxaloacetate (Scheme 1), rather than the result of increased utilization of aspartate in the adenylosuccinate synthase reaction.

It was found that hadacidin did not decrease cell respiration, indicating that there was no disturbance of ATP production owing to inhibition of the respiratory chain or other energetic processes in the mitochondria (results not shown).

DISCUSSION

Although it is generally accepted that glutamine oxidation is increased in malignant and normal rapidly dividing cells, there is still controversy about which pathway of oxidation is predominant. This problem is of importance if we are seeking the answer to the question of significance of glutamine oxidation for tumour-cell metabolism and cellular physiology in general. Most of

the experimental data indicate that the aspartate aminotransferase pathway predominates, although in some tumours alanine aminotransferase (EC 2.6.1.2) may be of importance also [14]. Several proposals about the significance of glutamine oxidation for tumour-cell energetics have been suggested. Although Newsholme's proposal [8], which emphasizes that glutamine oxidation is only partial and important for biosynthetic reactions rather than for cell energetics, has some experimental support, because glutamine oxidation leads to accumulation of intermediates such as aspartate and glutamate, it is clear that even this incomplete oxidation results in significant net production of ATP. This was supported very recently by Kelleher and collaborators [15], who provided evidence that AS-30D rat ascites-hepatoma cells possess a capacity for conversion of glutamine into tricarboxylic acid-cycle intermediates that is quantitatively significant relative to tricarboxylic acid-cycle flux. Also, they showed that fluxes to aspartate and alanine are major carbon exit paths from the tricarboxylic acid cycle. Further, it has been reported from several laboratories that NAD(P)-dependent malic enzyme (EC 1.1.1.39), which has increased activity in malignant cells, can ensure complete oxidation of glutamine [14,16,17].

Apart from these aspects of the role of glutamine oxidation in tumour energetics, there are no studies related to the possibility that the large production of aspartate may be essential for the re-synthesis of AMP in the purine nucleotide cycle. It has been demonstrated by Lowenstein and co-workers that the cycle is of importance for the energy metabolism of some normal tissues, especially skeletal muscle [18]. This metabolic pathway may consume a large amount of aspartate. Although the formation of AMP from IMP and aspartate is certainly important for biosynthetic reactions such as nucleic acid synthesis, it may be equally important for the energy metabolism of malignant cells. This is because malignant cells are frequently exposed to variable periods of hypoxia and ischaemia which cause acute metabolic stress. In the absence of O_2 and glucose (ischaemia) there is a rapid disappearance of ATP. In order to maintain a high ATP/ADP ratio, which is an index of the cellular energy state, AMP deaminase (EC 3.5.4.6) is activated [19]. Metabolic processes are much more sensitive to changes in the ATP/ADP ratio than to changes in the absolute concentration of ATP [20]. In order to maintain a sufficiently high ATP/ADP ratio, the cells will sacrifice part of the adenine nucleotides pool by activating AMP deaminase and coupling this reaction with the reaction of adenylate kinase. The extent of the decrease in total adenylate concentration probably depends on the factors which regulate the activity of AMP deaminase. This activity increases markedly with decrease of adenylate energy charge [20]. Reoxygenation should ensure not only rapid synthesis of ATP but also regeneration of the total pool of adenylates. This requires rapid resynthesis of IMP from inosine through the purine salvage pathway, and an adequate production of aspartate, mainly from glutamine oxidation (Scheme 1). Both these metabolic pathways are very active in malignant cells [10]. AMP is formed from aspartate and IMP in the reaction of adenylosuccinate synthase, whereas adenylate kinase equilibrates AMP with ADP and ATP.

Our experimental results strongly support the above proposal. The increase in the total adenine nucleotide pool under aerobic conditions in the presence of glutamine and inosine is certainly a consequence of resynthesis of AMP in the adenylosuccinate synthase reaction. Under anaerobic conditions there is depletion of a part of the pool, whereas the rest of the pool is very stable. If malignant cells existed under stable metabolic conditions, like normal cells, there would be no necessity for a large utilization of aspartate, because the pool of adenine nucleotides would be stable. However, frequent episodes of anoxia have as a consequence the utilization of substantial amounts of aspartate. This was demonstrated in the experiments in which ascites cells were alternately incubated with N_2 and O_2. This caused a marked decrease in the content of aspartate compared with the situation where hadacidin was added. Continuous aerobic incubation with hadacidin did not decrease aspartate. This suggests that the amount of aspartate which is channelled through AMP into nucleic acid synthesis is much smaller than that which is used for the resynthesis of AMP after frequent interchange of aerobic and anaerobic conditions. Concerning changes in the pool of adenine nucleotides, Mowbray and collaborators [21–23] isolated from rat heart and kidney an oligomeric adenosine tetraphosphate. They suggested that it may be a storage pool of adenine nucleotides and part of a homoeostatic mechanism for the ATP/ADP ratio, but its true function and existence in tumours is still unknown. Great numbers of malignant cells, especially when rapidly proliferating, probably exist on the border between aerobiosis and anaerobiosis. Adaptation to these conditions must be of particular importance not only for proliferation but also as the stimulus for tumour progression.

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