Proliferation Kinetics and Metabolic Features of *in vitro* Grown Ehrlich Ascites Tumor Cells in the Presence of Exogenous Pyruvate

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Ehrlich Ascites Tumor Cells, Growth Inhibition, Pyruvate, Metabolism

The proliferation of *in vitro* grown Ehrlich ascites tumor cells is inhibited by pyruvate concentrations >2 mM. In the presence of 4–5 mM pyruvate the growth is reduced to about 50%, in the presence of 20 mM to about 5–10%. Viability of the cells is not severely affected. Increase of DNA corresponds to the cell growth. On recultivation in pyruvate free standard medium, growth is nearly normal. Flow cytometric analyses of the proliferation kinetics of the cells in the presence of 20 mM pyruvate revealed a retardation of the passage of all phases of the cell cycle. No phase specific effects could be detected though the S- and G2M-phase are more afflicted than G1. The growth inhibition of EAT cells by pyruvate seems to depend on the presence of glucose.

Exogenous pyruvate (>1–2 mM) causes an activation of pyruvate dehydrogenase, a reduction of lactate production from glucose and a stimulation of lipid biosynthesis; the NAD/NADH ratio of the cells is reduced and a rise of glycolytic intermediates beyond glyceraldehyde-3-phosphate dehydrogenase is observed. Maximal activation of pyruvate dehydrogenase by non toxic concentrations of dichloroacetate is also accompagnied by an inhibition of cell growth. It is suggested that an increase of glyceraldehyde-3-phosphate level and the changes in the redox state of the cells are of relevance for the inhibition of cell growth by pyruvate. 100–500 μM exogenous glyceraldehyde-3-phosphate strongly inhibited cell growth.

It is becoming increasingly likely that pyruvate, a key anaplerotic metabolite, may play a regulatory role in cell proliferation. Supplements of pyruvate to tissue culture medium enhance the proliferation of certain types of mammalian cells in culture [1–3], especially when cell densities are low. In some types of cells an inverse relationship between levels of serum derived growth factors and the enhancement of cell proliferation by pyruvate and 2-oxo-carboxylates has been demonstrated [4–7]. Pyruvate not only stimulates the growth of cells in culture, but also affects their differentiation [8, 9].

Malignantly transformed cells have altered growth factor and nutrient requirements in culture [10, 11]. We therefore have studied growth and metabolism of Ehrlich ascites tumor cells in the presence of increasing concentrations of exogenous pyruvate. These experiments have shown that pyruvate is a growth inhibiting factor for these cells between 2–20 mM, which causes a retardation of the passage of all phases of the cell cycle. These effects of pyruvate depend on the presence of glucose.

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Exogenous pyruvate causes further a strong activation of pyruvate dehydrogenase, a reduction of lactate production from glucose and a stimulation of lipid biosynthesis; the NAD/NADH ratio is reduced and an increase of glycolytic intermediates beyond glyceraldehyde-3-phosphate dehydrogenase could be detected. We suggest that the rise of glyceraldehyde-3-phosphate and the changes of the redox state are relevant for the inhibition of cell proliferation by pyruvate.

Material and Methods

All chemicals, buffers and media components were of the purest grade available from Merck (Darmstadt), Serva (Heidelberg), Sigma (München) and Boehringer (Mannheim). L-U-[^{14}C]lysine (s.a. 287 Ci/mol), 2-[^{14}C]thymidine (s.a. 53–61 Ci/mol) were purchased from Amersham Buchler (Braunschweig). Scintillation cocktail Rotiszint R11 was from Roth (Karlsruhe). Horse serum was from Behringwerke Marburg, it was essentially free of mycoplasma. Microcillin was from Bayer (Elberfeld).

Cells and growth techniques

Hyperdiploid Ehrlich ascites tumor cells, strain ELT-Bonn [12] were serially grown in the peritoneal

cavity of female NMRI mice. Cells for explantation to cultures *in vitro* were usually withdrawn from mice inoculated 4–5 days previously and transferred to modified Eagle's medium supplemented with 15% horse serum, 30 mg/l streptomycin and 575 mg/l microcillin, to obtain a suspension of 5×10^5 cells/ml of culture medium in 600 ml non-siliconized glass flasks. Growth was estimated by counting the cells in a Neubauer chamber or by turbidity measurements. Viability of the cells was assessed by dye exclusion test with 0.1% nigrosin.

After 13–15 h cultivation at 37 °C under standard conditions (first passage *in vitro*) the cells were transferred to fresh culture medium. The inoculum density in the second and subsequent passages was $3.5–4.0 \times 10^5$ cells/ml. The culture flasks were slowly rotated for 2 min every 2 h with a frequency of 2 min^{-1}. Pyruvate was added to the culture medium from a 1 M solution of sodium pyruvate, which was prepared before every experiment.

Cell cycle analysis

Cell cycle distribution of the cell populations was analyzed by flow cytometry as described in [13] and [14]. The fluorescence of DNA bound dye was measured proportionally, stored and graphed with a flow cytometer ICP 11, Phywe Göttingen. The quantitative evaluation of the histograms to obtain the fractions of cells in the various compartments is illustrated in [13].

Measurement of metabolic parameters

Protein content of cell cultures was quantified by the Lowry method with cristallized bovine serum albumin (Behringwerke Marburg) as standard. DNA content of cell cultures was determined fluorometrically as described by Labarca and Paigen [15]. The relative rates of DNA and protein synthesis were measured by incorporation of 2-[^{14}C]thymidine and U-[^{14}C]lysine into acid insoluble fraction as described in [16]. L-Lactate production of the cells was assayed as described by Hohorst [17], glucose consumption was measured with the glucose oxidase period test from Boehringer (Mannheim).

ATP and ADP concentrations of the cells were assayed by applying the luciferin/luciferase system using a Biolumat 9500 (Bertholt). For further details see reference [13]. ATP and ADP in the cytosol were determined as described by Zuurendonk and Tager [18].

NADH and NAD were determined with a bioluminescence system as described by Stanley [19]. For determination of NAD 2 ml of cell suspension were deproteinized with 0.2 ml 3.3 N perchloric acid. The precipitate was separated by centrifugation and the supernatant was neutralized with 3 N KOH. 200 µl of the probe were utilized for the assay. For determination of NADH 1 ml of cell suspension was deproteinized with 0.5 ml 1 N alcoholic KOH. For further details of the method see [13, 19].

Total lipids of the cells were extracted from the perchloric acid precipitate with chloroform/methanol (1:1 V/V) as described by Folch [20] and determined gravimetrically (Cahn electro balance [21].

Intermediates of glycolysis (glucose-6-phosphate, fructose-1.6-bisphosphate, glyceraldehyde-3-phosphate and dioxyaceton-phosphate) were assayed by standard methods as described in [22]. 15 ml of cell suspension were deproteinized with 1 ml 5 N perchloric acid, after centrifugation the supernatant was neutralized with 5 M K_2CO_3 solution; 4 ml of the extract were used for the determination in a 4 cm cuvette [13].

Assay of pyruvate dehydrogenase in vivo

Pyruvate dehydrogenase activity of the cells *in vivo* was assayed via the $^{14}CO_2$ liberation from 1-[^{14}C]pyruvate. Two methods were applied; first a continuous measurement of produced $^{14}CO_2$ as was developed earlier in our laboratory [23] and further a discontinuous assay which provides only one single endpoint from each incubation vessel. In the last case essentially the method of Borud and Stromme [24] was applied; the experimental procedure is described in detail in [13] and [24]. 2 ml cell suspension were used for each analysis.

Results

Proliferation kinetics of the cells

In order to determine the dependency of growth response of EAT cells on increasing concentrations of pyruvate, we have compared the production of cells in culture within 24 h in the presence of 0.5–20 mM pyruvate. The results of these experiments have shown (see Fig. 1) a steady decrease of cell growth in pyruvate supplemented medium above a concentration of 1 mM. Viability of the cells is not significantly impaired up to 20 mM pyruvate. We observed further that concentrations of 0.5–1 mM py-

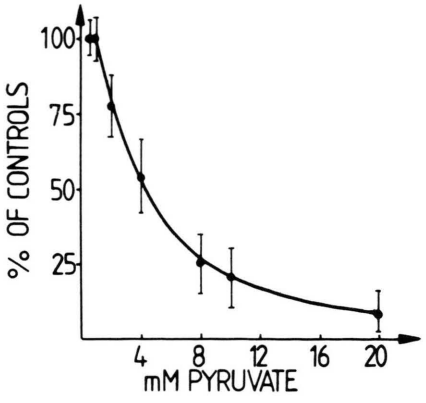

Fig. 1. Growth within 24 h of EAT-cells in the second passage *in vitro* in the presence of increasing concentrations of exogenous pyruvate. Cell density at the beginning of each experiment was $3-4\times10^5$ cells/ml; cell density of controls after 24 h was between $2-3\times10^6$ cells/ml; Glucose 10 mM.

Table I. Effect of pyruvate on cell proliferation of *in vitro* grown EAT cells in different culture media. Inoculum density was $3-4\times10^5$ cells/ml, cell density of controls after 24 h was between $2-3\times10^6$ cells/ml. Results of typical experiments.

Medium composition	Rel. growth % of controls within 24 h
10 mM glucose (Controls)	100
10 mM glucose + 20 mM pyruvate	5–10
glucose free	0
10 mM glucose + 10 mM uridine	110
glucose free + 10 mM uridine	40–60
glucose free + 10 mM uridine + 20 mM pyruvate	40
10 mM glucose + 10 mM uridine + 20 mM pyruvate	15

ruvate may not stimulate the proliferation of EAT cells even not at low cell densities (5×10^4 cells/ml). Results of experiments which demonstrate the significance of glucose for the growth inhibiting activity of pyruvate are summarized in Table I. In these experiments we have taken advantage of the previous observation, that growth and viability of EAT cells in glucose free medium can be sustained by uridine [25]. In glucose free medium supplemented with uridine, addition of 20 mM pyruvate does only slightly impair the growth of the cells. It seems further that uridine not only may stimulate the growth of the cells under normal conditions but also in the presence of pyruvate.

In further experiments we have characterized the growth inhibiting properties of pyruvate in more detail. The relative increase of cell number, DNA and protein within 24 h of a typical experiment are depicted in Fig. 2 A, B and C. The production of cells in the presence of 20 mM pyruvate was reduced to

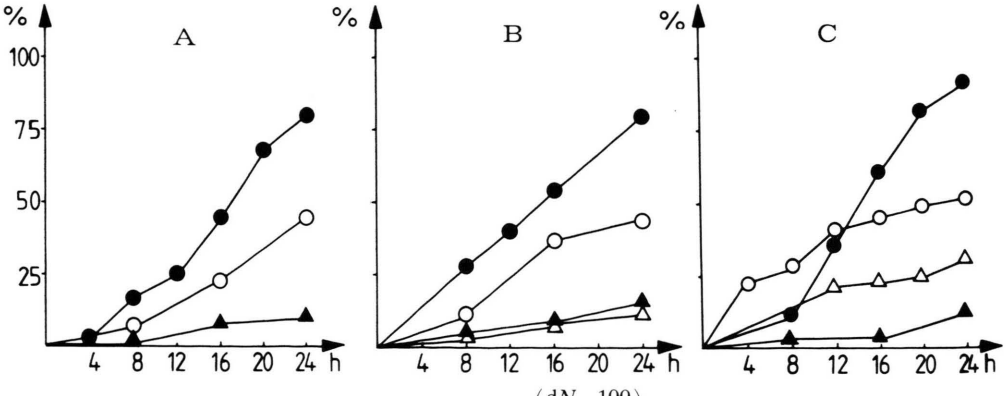

Fig. 2. A. Relative increase of cell number in percent $\left(\frac{dN}{dt}\ \frac{100}{No}\right)$. Controls –●– (inoculum density 3×10^5 cells/ml, Glucose 10 mM; +20 mM pyruvate –▲–; recultivation in pyruvate free medium (3rd passage) –○–.
B. Relative increase of DNA. Controls –●– (DNA: 11.9 μg/10^6 cells at zero time); +20 mM pyruvate –▲–; 3rd passage in the presence of 20 mM pyruvate –△– (DNA: 12.2 μg/10^6 cells at zero time); recultivation in pyruvate free medium (3rd passage) –○–. Cells were separated from 1 ml cell suspension, DNA was measured fluorometrically as described in (15).
C. Relative increase of protein. Controls –●– (at zero time 217 μg/10^6 cells); +20 mM pyruvate –▲–; 3rd passage in the presence of 20 mM pyruvate –△– (245 μg/10^6 cells); recultivation in pyruvate free medium (3rd passage) –○–. Estimation of protein with Lowry reagent after separation of the cells from 1 ml of cell suspension.

about 5–10% of controls within 24 h (Fig. 2A). In the third passage in the presence of the same pyruvate concentration the increase of cell number was in the same range. On recultivation in normal medium an increase of cells of about 60–80% of controls was obtained. Increase of DNA of the cultures corresponds to the increase of cell number as is demonstrated in Fig. 2B while the increase of protein (Fig. 2C) suggests, that the "growth cycle" of the cells is less impaired by pyruvate than the "chromosome cycle". The effects of 20 mM pyruvate on the rate of DNA- and protein synthesis of the cells are illustrated in Fig. 3A and 3B. A typical incorporation pattern of [^{14}C]thymidine was obtained under standard conditions, while in the presence of 20 mM pyruvate the DNA synthesis is dramatically reduced within 8 h and thereafter remains constant up to about 30 h. The rate of protein synthesis in the presence of 20 mM pyruvate (Fig. 3B) has already attained a minimum after 4 h and remains constant up to 30 h.

Cell cycle progression

In order to obtain further information on the proliferation kinetics of the cells, flow cytometric analyses of the phase composition of controls and of cultures grown in the presence of 20 mM pyruvate were performed. The results of a flow cytometric analysis of a typical experiment are summerized in Table II.

From the increase of cell number and a comparison of the phase composition at the beginning of the culture period and after 24 h, the following conclusions may be drawn: At the beginning of the experiment in the presence of 20 mM pyruvat 38 out of 100 cells are in G1, 42 in S and 20 in G2M. Since within 24 h cell growth was about 10%, at the end of the culture period 26 cells are in G1 (24% from 110), 52 are in S (47% from 110) and 32 are in G2M (29% from 110). Within 24 h only 10 from 20 cells being in G2M at the beginning of the experiment have divided (10% growth) and have entered the G1 phase, which implies that only half of the G2M cells have passed mitosis within 24 h, while the normal length of G2M is 7 ± 1 h [19]. During the same time 22 S-cells have entered G2M to yield 32 G2M cells. After 24 h 20 S-cells out of 42 are yet in their compartment; the normal length of S is 9 ± 1 h. Within the culture period 32 G1 cells enter the S-phase to yield 52 S-cells; 26 cells are in the G1-compartment at the end of the culture period. From 38 cells being in G1 at the beginning of the experiment, 32 leave the phase within 24 h; the normal length of G1 is

Table II. Cell cycle distribution of EAT-cells grown in the presence and absence of pyruvate.

Controls				+ 20 mM Pyruvate		
h	Gl	S	G2M	Gl	S	G2M
0	41	39	20	38	42	20
12	26	44	30	23	56	21
24	22	45	33	24	47	29
$\frac{dN}{dt} \cdot \frac{100}{No}$ in 24 h: 70%				$\frac{dN}{dt} \cdot \frac{100}{No}$ in 24 h: 10%		

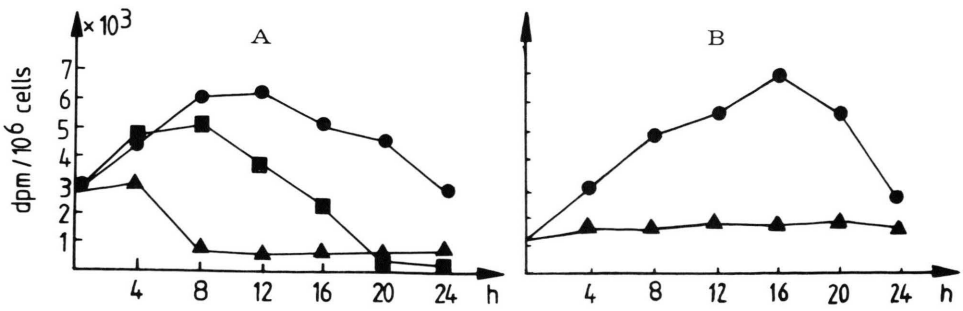

Fig. 3. A. Incorporation of [^{14}C]thymidine into EAT cells after a 15 min puls of 0.1 μCi/ml. Controls –●–; in the presence of 10 mM dichloroacetate –■–; in the presence of 20 mM pyruvate –▲–. Cell density between $3.5-4 \times 10^5$ cells/ml, glucose 5.5 mM.
B. Incorporation of U-[^{14}C]-L-lysine after a 15 min puls of 0.1 μCi/ml. Cell density between $3.5-4 \times 10^5$ cells/ml, glucose 5.5 mM. Controls –●–, 2nd passage in the presence of 20 mM pyruvate –▲–.

18 ± 1 h. From these estimations it is concluded that pyruvate causes a drastic retardation of all phases of the cell cycle, the G1 phase being less afflicted than the S- and G2M compartment. From the growth of the control cultures a generation time of 32 h may be estimated [26].

Metabolic features of the cells

The most prominent alteration of energy and intermediary metabolism in the presence of exogenous pyruvate is a considerable increase of pyruvate dehydrogenase activity (Fig. 4). Within the first 6 h a stimulation of the enzyme up to 15–20-fold was measured; the activity runs through a maximum and attains 600% of controls after 24 h. The same effect on the enzyme activity *in vivo* was detected in the presence of 10 mM dichloroacetate, a specific activator of pyruvate dehydrogenase [27]. Nontoxic concentrations of pyruvate or dichloroacetate, which give rise to a maximal activation of pyruvate dehydrogenase, have qualitatively the same but quantitatively different influences on proliferation and DNA synthesis (Fig. 3A). Proliferation of the cells in the presence of 10 mM dichloroacetate is reduced by about 50% of controls within 24 h (data not shown).

Glucose consumption and lactate production are illustrated in Table III. This table demonstrates that activation of pyruvate dehydrogenase reduces the glucose uptake and lactate formation of the cells. Pyruvate dehydrogenase activity and total lipid synthesis in the presence of increasing concentrations of exogenous pyruvate are compared in Fig. 5. This figure demonstrates that the incorporation of ^{14}C from U-[^{14}C]pyruvate into total lipids of the cells and the pyruvate dehydrogenase activity correspond very

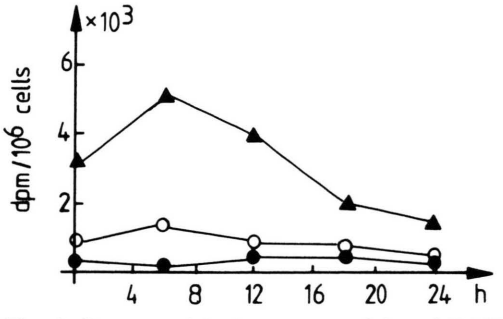

Fig. 4. Pyruvate dehydrogenase activity of EAT-cells *in vivo*. Controls –●–, cells grown in the presence of 20 mM pyruvate or 10 mM dichloroacetate –▲–, cells treated with 20 mM pyruvate in the 2nd passage and recultivated in standard medium (3rd passage) –○–. Parameter of activity is $^{14}CO_2$ liberated from 1-[^{14}C]pyruvate; s.a. of 1-[C^{14}]pyruvate was 14.4 Ci/mol, activity in the incubation medium (4 ml) was 1 μCi, cell density was 2×10^6 cells/ml; the first point was obtained 4 min after addition of 20 mM pyruvate. Activation of the enzyme is already detectable after this time. For further details see "Methods".

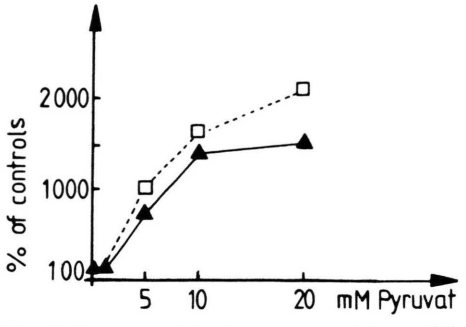

Fig. 5. Pyruvate dehydrogenase activity and lipid synthesis in the presence of increasing concentrations of exogenous pyruvate. $^{14}CO_2$ evolution from 1-[^{14}C]pyruvate –□–; conditions see Fig. 4. ^{14}C incorporation into total lipids from U-[^{14}C]pyruvate –▲–. Measurements after 90 min. EAT cells from the first passage were used. Cell density was $2-4 \times 10^6$ cells/ml; s.a. of U-[^{14}C]pyruvate was 14 Ci/mol.

Table III. Glucose uptake and lactate production of EAT cells between 4 and 24 h of the second passage *in vitro*. Glucose concentration 10 mM.

Conditions	Glucose uptake μmol/10^6 cells × h	Lactate production μmol/10^6 cells × h	*n*
Controls	0.431 ± 0.058	0.694 ± 0.082	32
+ 20 mM pyruvate	0.361 ± 0.047	0.524 ± 0.073	12
10 mM dichloro acetate	0.380 ± 0.062	0.578 ± 0.061	10
recovery (after 20 mM pyruvate)	0.412 ± 0.073	0.557 ± 0.098	8

well. Activation of the enzyme and an increased level of acetyl CoA (0.172 nmol/10^6 cells in controls, 0.299 nmol/10^6 cells in the presence of 20 mM pyruvate) give rise to a strong stimulation of lipid synthesis.

Data on the energetic state of the cells are given in Table IV. The ATP/ADP ratio of the whole cells under all conditions is 5.9 ± 1.0, in the cytoplasma 14.6 ± 0.8. The energy supply of the cells in the presence of exogenous pyruvate and dichloroacetate is not significantly impaired.

While the NAD/NADH ratio of dichloroacetate treated cells (specific activation of pyruvate dehydrogenase) is not changed as compared to controls, it is significantly reduced during and after growth in pyruvate supplemented medium (see Table V). Activation of pyruvate dehydrogenase does therefore not primarily account for the shift of the redox state in the presence of exogenous pyruvate. Other metabolic effects must be taken into consideration (see discussion).

Analyses of the intermediates of glycolysis have given further information. These experiments were undertaken because it is known that pyruvate is an inhibitor of lactate dehydrogenase [28] and pyruvate kinase [29]. Indeed an accumulation of metabolites above the glyceraldehyde-3-phosphate dehydrogenase was observed (see Table VI) which possibly is of

Table IV. ATP-, ADP-content and ATP/ADP ratio of the cells between 4 and 24 h of the second passage *in vitro*.

Conditions	ATP nmol/10^6 cells	ADP nmol/10^6 cells	ATP/ADP	*n*	
Controls	6.86 ± 1.02	1.43 ± 0.26	4.79	90	whole cells
+ 20 mM pyruvate	7.27 ± 1.07	1.08 ± 0.34	6.73	30	
+ 10 mM dichloro acetate	6.08 ± 0.71	1.17 ± 0.26	5.19	24	
recovery (after 20 mM pyruvate)	6.68 ± 0.72	0.97 ± 0.31	6.88	36	
Controls	6.49 ± 1.21	0.48 ± 0.99	13.5	18	cytoplasma
+ 20 mM pyruvate	7.72 ± 1.25	0.497 ± 0.99	15.44	18	
+ 10 mM dichloro acetate	6.55 ± 0.96	0.447 ± 0.085	14.88	18	

Table V. NADH-, NAD-content and NAD/NADH ratio of EAT cells between 8 and 24 h of the second passage *in vitro*.

Conditions	NADH nmol/10^6 cells	NAD nmol/10^6 cells	NAD/NADH	*n*
Controls	0.336 ± 0.070	6.50 ± 1.00	19.3	29
+ 20 mM pyruvate	0.884 ± 0.080	6.85 ± 1.14	7.7	24
+ 10 mM dichloro acetate	0.496 ± 0.082	9.04 ± 1.32	18.3	18
recovery (after 20 mM pyruvate)	0.750 ± 0.146	6.50 ± 1.18	8.7	18

Table VI. Concentration of intermediates of glycolysis of *in vitro* grown EAT cells between 4 and 24 h.

Metabolite	Controls nmol/10^8 cells	+ 20 mM pyruvate nmol/10^8 cells	*n*
Glucose-6-phosphate	116 ± 32	155 ± 29	5
Fructose-2.6-bisphosphate	64 ± 21	169 ± 35	5
Glyceraldehyde-3-phosphate	71 ± 18	198 ± 42	5
Dioxyaceton-phosphate	65 ± 19	179 ± 37	5

relevance with regard to the growth inhibition of cells in pyruvate medium. We have focussed our interest on glyceraldehyde-3-phosphate, because Fenselau *et al.* [30] have shown that aldosephosphates are inhibitors of cell growth and especially that glyceraldehyde-3-phosphate is a potent inhibitor of the growth of 3T3 cells.

Our experiments have demonstrated, that 100–500 μM glyceraldehyde-3-phosphate inhibit severely the growth of EAT cells without impairment of viability. The analysis of the proliferation kinetics of these cultures (data not shown) revealed in a typical experiment, that only cells which are at the beginning of the experiment in mitosis divide and enter G1 (3%). About 5% of G1 cells enter the S-phase and about 8% of S-cells enter G2 within 24 h. The small increase of cell number and the slow progress of cells through the cell cycle resemble the proliferation kinetics of the cells in the presence of pyruvate.

Discussion

The concentration dependent growth inhibiting effect of pyruvate on EAT cells is somewhat surprising, since pyruvate and other 2-oxoacids are known to promote the proliferation of *in vitro* grown cells of different types [4–7]. These cell types include also malignant cells as was shown for instance for Walker Carcino-sarcoma cells [1]. To our best knowledge there exists only one report on an inhibiting effect of pyruvate on cells *in vitro:* differentiation of mouseterato/carcinoma cells is restricted by pyruvate [9]. The actual concentration of pyruvate required for maintaining inhibition of growth of EAT cells is above 1–2 mM.

The growth inhibition by pyruvate of EAT cells seems to depend upon the presence of glucose in the culture medium as is suggested by the experiments in glucose free medium supplemented with uridine. This finding is consistent with the assumption that intermediates of glycolysis may play a role as growth inhibiting factors, because glycolysis in glucose free uridine containing medium is reduced by more than 90% [25]. The flow cytometric analyses of the cell cycle kinetics in the presence of 20 mM pyruvate revealed that only a fraction of the cells being in G2M at the beginning of the culture period enter mitosis and divide within 24 h, no G1- nor S-cells pass the cell cycle during this time. It is evident that no complete arrest of cell growth takes place, obviously even not in the second passage in the presence of high concentrations of pyruvate. The increase of DNA and of protein confirms a slow progress of the cultures through the cell cycle, as does the rate of DNA and protein synthesis. In the presence of lower concentrations of pyruvate, a corresponding smaller retardation of cell cycle progression is observed (data not shown).

One of the most interesting aspect of these studies is the relation between cell proliferation, rate of aerobic glycolysis (lactate production) and pyruvate dehydrogenase activity, since it is well known that high malignancy (low degree of differentiation and fast growth) is always combined with high aerobic glycolysis and low pyruvate dehydrogenase activity [31].

It appears that pyruvate potentially may influence the intermediary metabolism and glycolytic/mitochondrial energy production via the following metabolic processes: activation of pyruvate-dehydrogenase by inhibiting pyruvate dehydrogenase kinase; reduction of lactate production (aerobic glycolysis) by inhibition of pyruvate kinase [29] and lactate dehydrogenase [28] and inhibition of glutamate-oxalacetate transaminase, which is part of the malat-spartat shuttle of the transport of cytosolic hydrogen into mitochondria [32].

A global function which pyruvate could modulate is the redox state of the cells [33]. Millimolar concentrations could raise the NAD/NADH ratio by acting as an electron sink for appropriate NADH oxidoreductases. Stimulation of cell proliferation and a more oxidizing state (higher NAD/NADH ratio) have been associated in some mammalian cells [34] although the correlation is not universal [35] and may depend on cell type. While an increase of NAD by pyruvate and other 2-oxocarboxylates would possibly enhance glycolysis and increase ATP levels, recent evidence from our laboratory suggests, that the electron flow per se is more important for cell cycle progression and cell's transition from G1 to S-phase than is the production of ATP therefrom [36].

A fall in the NAD/NADH ratio (raise of NADH) as is observed in EAT cells under the influence of pyruvate in our experiments is associated with a decrease of glycolysis and inhibition of cell growth. The raise of the NADH level only transiently enhances the oxygen consumption; over 24 h there was no increase in oxygen uptake in the presence of pyruvate as compared to controls [13]. This is not surprising

because we proceed from the assumption that the aspartate-malate shuttle of cytosolic hydrogen transfer into mitochondria is impaired in the presence of high concentrations of the 2-oxoacid. Dionisi *et al.* [37] have shown, that in the presence of aminooxyacetate, a specific inhibitor of amino transferases, the NAD/NADH ratio of EAT cells is reduced. As we have shown [13] 2 mM aminooxyacetate also blocks the growth of EAT cells. Inhibition of lactate dehydrogenase by pyruvate is a further reason for the increase of the NADH level of the cells. Cell proliferation and a more reducing state (decreased NAD/NADH ratio) seem not to be compatible. Activation of pyruvate dehydrogenase (enhanced production of acetyl CoA) and a more reducing state (raise of cytosolic NADH) stimulate however the lipid biosynthesis of the cells.

Experiments of Olivotto *et al.* [38] have shown, that the incorporation of [^{14}C]thymidine and [^{3}H]lysine into DNA and protein of Yoshida AH 130 hepatoma cells is inhibited by pyruvate. These authors have also observed that the inhibition could be completely reversed by addition of adenosine. In our experiments this nucleoside had no effect on the inhibition of cell proliferation of EAT cells by pyruvate, rather the inhibitory activity was enhanced [13].

Acknowledgements

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