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^{31}P-NMR-SPECTROSCOPY MEASUREMENTS OF ENERGY METABOLISM OF IN VIVO GROWING ASCITES TUMOURS FOLLOWING ADDITION OF GLUCOSE

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Abstract

The cellular ATP content and the phosphorylation potential, defined as the ATP, ADP and inorganic phosphate (Pi) ratios, of exponentially growing Ehrlich ascites tumour cells were compared with cells at the plateau phase of growth. These phosphorus compounds were measured using ^{31}P-NMR-spectroscopy immediately after removal of the cell material from the host and in their ascites fluid reflecting in vivo growth conditions. Reaching the plateau phase of growth, the ATP content and the phosphorylation potential decreased. Upon addition of glucose, the phosphorylation potential immediately increased. We concluded that the reduced phosphorylation potential was due to a limited availability of glucose in spite of the nearly normal blood glucose concentration found. An increasing diffusion distance from the host to all parts of the tumour is a possible reason for that.

Key words: Ehrlich ascites tumour, ^{31}P-NMR-spectroscopy, energy metabolism.

After a certain time *in vivo* growing ascites tumour cells reach a plateau phase of growth. Different mechanisms for growth inhibition have been discussed: e.g. environmental factors, such as deprivation of nutrition (1), decreased glucose concentration (2) or decreased O_2 pressure (3, 4); the existence of autoregulative mechanisms which is supported by the regrowth of the remaining cells after aspiration of a part of the tumour cell population (5); the correlation between size of the tumour and the total number of cells at the plateau phase (6–11). Furthermore, when a second tumour is transplanted into an ascites tumour at the plateau phase of growth, this second tumour also grows up to a plateau phase resulting in about a doubling of the total number of cells (12). The metabolic activity of the growth inhibited cell is characterized by a depressed macromolecular synthesis (13, 14).

In the present investigation we found a decreased cellular ATP content of *in vivo* growing Ehrlich ascites tumour cells reaching the plateau phase of growth. In order to elucidate the reason for this decreased ATP content the ability of these cells to synthesize ATP after addition of glucose was studied using ^{31}P-NMR-spectroscopy. Based on these results, lack of glucose as a significant factor for the growth inhibition at the plateau phase of growth can be discussed.

Material and Methods

Cell material and experimental procedures. A number of 30×10^6 hyperdiploid Ehrlich ascites tumour cells 7–10 days old were injected i.p. into 3-month-old female NMRI-mice.

The total number of cells at various times after inoculation was obtained from the ascites volume and the cell concentration. The ascites volume was determined from the dilution of i.p. injected ^{125}I-albumin of 200000 CPM in 0.2 ml. The cell concentration of the ascites cell suspension was calculated in a Bürker chamber and the cell viability was determined by dye exclusion test. The viability was more than 98%. (For further details, see reference 15.) The cellular ATP content was measured using the luciferin-luciferase technique immediately after removing the cells from the host (16). After precipitation in 0.33 mol/l PCA the glucose concentration in the blood and in the ascites fluid was determined using a test-combination gluco-quant kit from Boehringer Mannheim GmbH diagnostica (17).

After the mice were killed by cervical dislocation, 3 ml

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of ascites cell suspension from 2 animals with a final concentration of about 250×10^6 cells per ml was removed by pre-chilled pipettes and put into pre-chilled NMR tubes without any washing procedures. The tubes were put into the NMR spectrometer and the measurement started 2 to 3 min after killing of the animals. The measurements were performed at a temperature of 7°C. When feeding the tumour cells, 0.1 ml of glucose was added to the ascites cell suspension in the NMR tubes to a final concentration of 30 mmol/l 1–2 min before starting the NMR measurements, when the temperature of the tumour was still 37°C or just below. No changes in the ATP content according to transferring and storage in the NMR-tubes was found (Table 1).

In experiments designed to study the saturation effect in the NMR spectrum exponentially growing cells were fed with glucose to a final concentration of 30 mmol/l just before measurements in order to prevent starvation during the experimental run.

The phosphorus compounds in the ascites fluid were measured using the ^{31}P-NMR-spectrometer after centrifugation of the ascites cell suspension at 500×g for 10 min. The same parameters were applied as for the measurement of the cell suspension.

NMR-measurements. All NMR-spectra were recorded on a Varian XL-300 spectrometer at a ^{31}P-frequency of 121.4 MHz. Typical 512 45° pulses with a repetition time of 1 s were used resulting in 8.5 min measuring time for each sample. Because of the relatively broad lines in the spectrum, short experimental times and high magnet stability it was not necessary to use field-frequency stabilization, such as internal deuterium loch.

In each spectrum individual peak areas were measured by planimetry to use peak area ratios as relative concentration ratios of the compounds studied. However, these area ratios were not equivalent to concentration ratios due to partial saturation of some peaks with slow relaxing components (18–20) caused by the relatively fast pulse repetition rate (when compared to the expected range of spin-lattice relaxation times). In order to quantify these saturation effects a separate experiment was performed. Measurements with different pulse-repetition rates (1 and 10 s respectively, otherwise identical conditions, the relaxed spectrum) were compared to provide estimations of scaling or saturation factors. Of the components relevant to this study only the inorganic phosphate (Pi) peak needed to be corrected by a factor of 2.0. Thus, in the results of each sample corrected peak–area ratios were used. The signals were corrected for the noise by subtracting the half height of the noise at corresponding peaks.

Results

Cell growth. The tumour cells were growing exponentially up to 5 days after transplantation and reached the plateau phase 3 days later (Fig. 1).

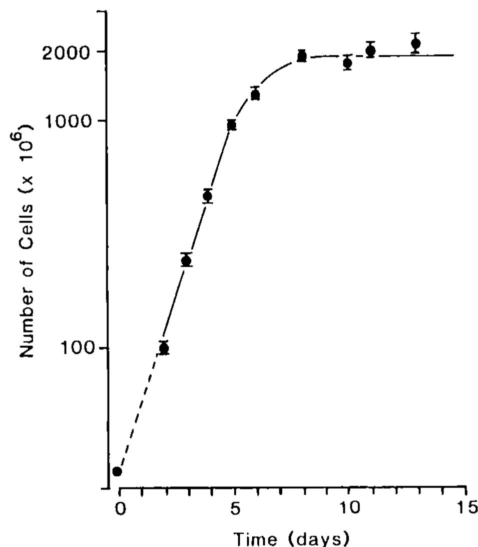

Fig. 1. Total number of tumour cells during the growth. Mean values±SEM of 5 to 8 animals.

Table 1

The ATP content of tumour cells as treated as in the case of NMR-measurements. The ATP was determined by luceferin-luceferase assay. Mean value of 6 animals±SE

Time (min)	ATP fmol/cell
0	4±2
3	5±0.6
6	4±0.2
9	4±1
12	6±0.8
30	4±1

^{31}P-NMR-spectra of exponentially growing and growth-inhibited cells. Examples of ^{31}P-NMR-spectra from various stages of the tumour growth are shown in Fig. 2. The assignments of the peaks were made by running spectra of pH-adjusted standards of ATP, ADP, AMP and NAD. Literature data (20) confirmed this identification and provided tentative assignment of the resulting peaks. The shift scale (in ppm) used was not related to any shift standard and is only a measure of relative peak positions within each spectrum. The peak to the left (28.5–30.5 ppm) contains mainly sugar phosphates but also AMP and phosphorylcholine. The peak at 27.7 ppm is inorganic phosphate, the 2 peaks at 26.4 and 25.9 ppm are glycero-3-phosphoryl-ethanolamine and glycero-3-phosphorylcholine respectively. The peaks at 20.3 and 19.6 ppm are γ-ATP and β-ADP, and the peaks at 15.4 ppm contain both α-ATP and α-ADP. The peaks at 20.3, 19.6 and 15.4 ppm also include other nucleotides but their concentrations are negligible. The peak at 14.7 ppm con-

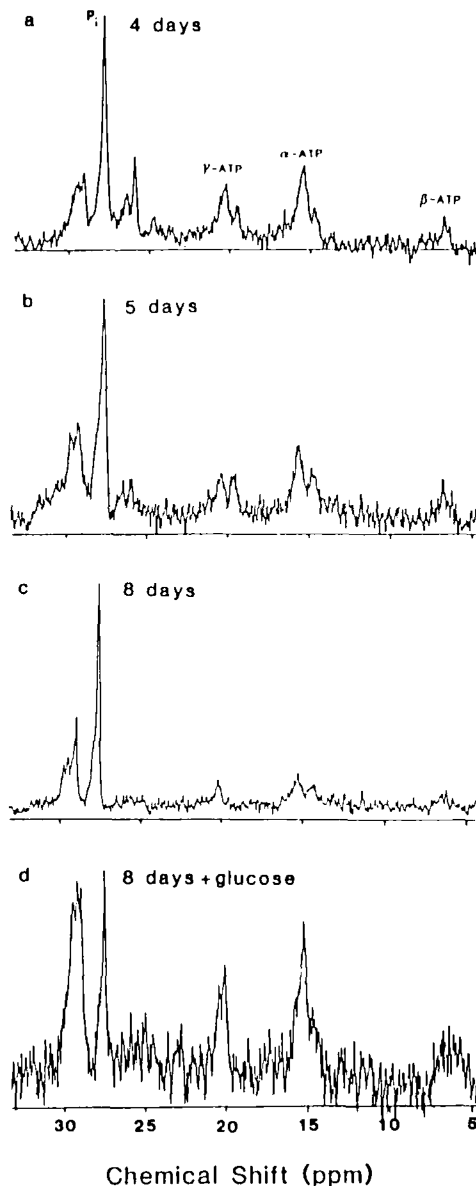

Fig. 2. Representative examples of ^{31}P-NMR-spectra ascites tumour cells at different times during the growth. (For complete assignment see the text). a) 4 days after inoculation. b) 5 days after inoculation. c) 8 days after inoculation. d) 8 days after inoculation following addition of glucose.

tains mainly NAD^+ and NADH, but even other compounds. The peak to the right at 6.7 ppm represents β-ATP from which the amount of ATP was determined.

In ascites fluid no detectable amounts were found of adenosine nucleotides and inorganic phosphates.

The ratios between ATP:ADP:Pi and thus the phosphorylation potential changed already during the exponential growth and decreased from 3.2 at day 4 to 1.2 at day 8 (Table 2).

Glucose concentration in blood and ascites fluid and cellular ATP content. The concentration of glucose in the blood of non-tumour bearing animals was 144 mg% (Table 3). Four to 5 days after inoculation of the tumour the amount of glucose decreased to 60 mg% but increased again when the tumour reached the plateau phase of growth. The amount of glucose in the ascites fluid was low (0.2–0.4 mg%) both during the exponential growth and the plateau phase of growth.

The cellular ATP content decreased by about 60% when the tumour reached the plateau phase of growth (Table 3).

Cellular ATP content following addition of glucose. In plateau phase cells, the addition of glucose resulted in a dramatical increase in the ATP:Pi ratio from 0.04:1 to 0.45:1 corresponding to an increase in the phosphorylation potential to about 8 times (Fig. 2d, Table 2). In the exponentially growing cells addition of glucose increased the ATP:Pi ratio from 0.19:1 to 0.71:1 resulted in a doubling in the phosphorylation potential (Table 2). Since the quantitation of the amount of ATP from the NMR-spectra was somewhat uncertain, particularly of the growth inhibited cells, and the values from the NMR-spectra were relative values, we measured the absolute amount of ATP by luciferin-luciferase assay. We found an increase in the cellular ATP content of the growth inhibited cells from 3.2±0.4 to 4.5±0.4 fmol/l per cell but no significant changes of the exponentially growing cells (from 8.3±1.0 to 7.5±1.1 fmol per cell). The results were mean values of 6 animals SE.

Discussion

^{31}P-NMR-spectroscopy has been used in order to study the energy production of normal growing cells, of cells in certain diseases and of tumour cells following treatment (18–20). NMR-spectroscopy has the advantage of being non-invasive. Cells and tissue can therefore be studied without any changes in their environment. This is important since procedures, such as washing and centrifuging of cell material, reduce the level of ATP, the utilization of glucose and the respiration rate by about 30% (21). Furthermore, precipitation of cell material using perchloric acid and/or freezing in liquid nitrogen changes the ratio between the phosphorus compounds of intact cells severely (17), a result which has been observed previously (18–20).

In the present study we removed the ascites cell suspension from the animals since a widebore NMR-spectrometer for *in vivo* studies was not available. Since the tumour cells were immediately transferred to pre-chilled NMR tubes and the accumulation time was relatively short, the metabolic state of the cells can be assumed to correspond well to the *in vivo* growth conditions. This assumption is further supported by earlier experience in which no change in the ATP content was found during incubation of ascites tumour cells up to 60 min (16).

We found an ATP:ADP:Pi ratio of 0.19:0.06:1 in exponentially growing cells. A similar ATP:ADP ratio in

Table 2

The relative concentration of inorganic phosphate (Pi) ADP and ATP and the phosphorylation potential at different times after inoculation and after addition of glucose at day 4 and day 8. Mean values of 2 to 20 animals±SE

Time after inoculation (days)	n	Relative concentration of			Phosphorylation potential $\frac{ATP}{ADP \times P_i}$
		ATP	ADP	P_i	
4	20	0.19±0.018	0.06±0.007	1	3.2
5	4	0.12±0.003	0.09±0.003	1	1.3
8	2	0.04±0.02	0.03±0.02	1	1.2
8+glucose	2	0.45±0.37	0.07±0.01	1	10.3
4+glucose	8	0.71±0.011	0.10±0.033	1	7.1

Table 3

Glucose concentrations in blood and ascites fluid and ATP content of the tumour cells at various days after inoculation of the tumour. Mean values of 4 to 9 animals±SE

Time (days)	Glucose (mg/100 ml)		ATP (fmol/l/cell)
	Blood	Ascites fluid	
Control	144±3	–	–
4–5	60±8	0.2±0.02	1.9±0.2
11–15	112±8	0.4±0.05	0.8±0.1

ascites tumour cells was also found using ^{31}P-NMR-spectroscopy (22). Using conventional biochemical procedures, higher ratios (0.4:0.1:1) have been reported (23, 24). These studies were, however, performed *in vitro* using glucose and an oxygen supply. The lower ratios found in the present study are therefore well explained by the anaerobic conditions known to be present in *in vivo* growing ascites cells (3, 4).

When the ascites tumour cells reached the plateau phase, the ATP:ADP:Pi ratio decreased corresponding to a decline in the phosphorylation potential of about 3 times. This is due to a dephosphorylation of ATP resulting mainly in an increase in the amount of phosphorus but also a decrease in the ATP content. Since the macromolecular synthesis was found to be depressed at this growth state (13, 14) the decrease in the phosphorylation potential seems not to be explained by an increase in the energy consumption. The energy consumption of the macromolecular synthesis was calculated to be about 40% of the energy produced (16).

The addition of glucose resulted in an increase in the phosphorylation potential of both plateau phase cells and exponentially growing cells. This indicates that also during exponential growth the ATP synthesis is not working at the maximum rate probably due to a limited availability of glucose.

The volume of glucose added (0.1 ml) was negligible in relation to the volume of the ascites cell suspension (3.0 ml). Thus, the elevated metabolic activity of cells after addition of the glucose cannot be caused by dilution of any growth inhibitor factors. Furthermore, since the phosphorylation potential was elevated immediately after addition of glucose, impairment of glycolysis cannot be responsible for the reduced phosphorylation potential found at the plateau phase of growth.

These results indicate that lack of glucose could be responsible for the reduced phosphorylation potential and thus inhibition of macromolecular synthesis and cell growth. A decrease in the amount of blood glucose seems, however, not to be the direct reason for the reduced ATP content since the concentration of the blood glucose was not related to the growth of the tumour. A reasonable explanation would be that when the tumour volume increases with age the capacity to feed all parts of the tumour decreases. Lack of glucose has also been discussed by others as a limiting factor for tumour growth when the tumour has reached a critical size (25). However, they concluded that the amount of glucose available in the host was the limited factor, not the way of feeding the tumour.

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