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Different Energy Metabolism in Two Human Small Cell Lung Cancer Subpopulations Examined by ^{31}P Magnetic Resonance Spectroscopy and Biochemical Analysis *in Vivo* and *in Vitro*[1]

Paul E. G. Kristjansen,[2] Mogens Spang-Thomsen, and Bjørn Quistorff

Department of Oncology, Finsen Institute-Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen [P. E. G. K.]; Institute of Pathological Anatomy, University of Copenhagen, Fred. d. 5. vej 11, DK-2100 Copenhagen [M. S-T.]; and Department of Biochemistry A, NMR Center, Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen [B. Q.], Denmark

ABSTRACT

Two human small cell lung cancer tumor lines, maintained as solid tumor xenografts on nude mice and as *in vitro* cell cultures, were studied by *in vivo* ^{31}P magnetic resonance spectroscopy and by biochemical analysis of extracts of solid tumors and cell cultures. The tumor lines CPH SCCL 54A and CPH SCCL 54B are subpopulations from the same tumor. In solid tumors ($n = 125$), the ATP/P_i ratio was greater in 54A than in 54B. This was due to a higher ATP level in 54A, whereas there was no difference in P_i, ADP, and AMP. A decrease in ATP/P_i during growth was caused by a decline in ATP, whereas P_i remained unchanged. Small amounts of phosphocreatine were found in the xenografts and in tumor extracts, but not in the cell extracts; correspondingly, there was a low creatine kinase activity in solid tumors and no activity in the cell cultures. Thus, the phosphocreatine content of the solid tumors originated from the stroma. A difference in ATP content between 54A and 54B was also found in cell cultures; hence, the metabolic difference is an intrinsic quality of the malignant cells and is not caused by the host system.

INTRODUCTION

Noninvasive ^{31}P-MRS[3] allows repeated analysis of tumor tissue *in vivo*, and the results provide information about tumor metabolism. Such information is potentially important in the characterization of malignant tissues and for monitoring of therapeutic effects (see Refs. 1–3 for a general review). Since quantitation of metabolites is difficult to obtain by ^{31}P-MRS, ratios of metabolites (*e.g.*, ATP/P_i) rather than absolute concentrations are usually presented. There is no unequivocal interpretation of differences or changes in steady-state metabolic ratios in tumors during growth or at therapy. In order to investigate this problem, a strategy was undertaken of using ^{31}P-MRS in combination with quantitative analytical biochemistry to analyze the energy metabolism in malignant tissue. Two human SCLC subpopulations, SCCL CPH 54A and 54B, derived from the same patient tumor and maintained both as solid tumor xenografts on nude mice and as *in vitro* cell cultures, were studied. We have previously reported a different metabolic response to irradiation in the two tumor subpopulations (4). The present study focuses on metabolic differences between the same two tumor lines during untreated growth using the combined methodology. In addition to the analysis of solid tumors, a corresponding analysis was performed on *in vitro* cell cultures of the same tumor lines to clarify whether the metabolic differences could be ascribed to the tumor cells themselves or whether they were produced by the mixed compartments of tumoral and stromal cells in the solid tumors. We found a significant difference in solid tumor ATP between the two SCLC subpopulations, which also was present when *in vitro* tumor cell preparations were compared, indicating that this difference was caused by intracellular factors. Furthermore, it was demonstrated that the amount of phosphocreatine present in the solid tumors is derived from the nonmalignant stromal cells.

MATERIALS AND METHODS

Cells, Tumors, and Transplantation

The tumor lines CPH SCCL 54A and 54B were derived from the same patient by *in vitro* cloning as described elsewhere (5). The original tumor was classified as an intermediate-type SCLC (6), and the histological characteristics have not changed during serial transplantation of 54A and 54B on nude mice. The two cell populations are distinguishable by their different DNA index, but otherwise they are similar. The DNA index is defined as the G_1 phase DNA content in the tumor divided by the normal human diploid DNA content (7). Chromosomal analysis has shown that cell lines 54A and 54B are copies of each other, except that 54A has doubled its DNA content (5). Accordingly, most features, *e.g.*, morphology and proliferation kinetics, are almost identical in the two tumor lines (5, 8), but their *in vivo* radiosensitivity is different; 54A is much more sensitive than 54B (8).

The tumors were maintained as xenografts in specific pathogen-free nude mice of NMRI and BALB/c background (Bommice, Ry, Denmark), kept under sterile conditions in laminar air flow benches. The room temperature was 25 ± 2°C, and the relative humidity was 55 ± 5%. Sterile food and water were given *ad libitum*.

The tumors were transplanted in the flanks of the animals as previously described (4), and normalized growth curves (9) were constructed to ensure unchanged growth in comparison with previous experiments. DNA flow cytometry was performed regularly to ensure stability of the DNA index.

In vitro cultures of 54A and 54B were serially grown in 150 cm^3 culture flasks (Flow) in Eagle's minimal essential medium containing Earle's salt supplemented with 10% fetal bovine serum, minimal essential medium amino acids and vitamins, L-glutamine, and glucose, without the use of antibiotics. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Experimental Design

Solid Tumor Xenografts. An initial small series ($n = 12$) of six tumors of each type was repeatedly analyzed by weekly *in vivo* ^{31}P-MRS, during the period 3–8 weeks following transplantation. The resulting series of spectra was analyzed for changes over time. The primary purpose of this experiment was to optimize the probe and to define the settings for subsequent experiments.

There was a difference in the spectra from 54A and 54B tumors, and this difference was further investigated in a second series of 47 tumors, analyzed in constant settings within 25–35 days after transplantation. Some of the spectra served as pretherapeutic controls in a previous study (4), in which the tumors were subsequently treated by irradiation.

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: ^{31}P-MRS, ^{31}P magnetic resonance spectroscopy; CK, creatine kinase; PCr, phosphocreatine; SCLC, small cell lung cancer.

In a parallel experiment, 66 tumors were freeze clamped on day 25, 30, or 60 after transplantation, perchloric acid extracted and analyzed for their metabolite content (ATP, PCr, ADP, AMP, and P_i), and subsequently examined for any difference between time points and tumor type. By this procedure, contamination with murine tissues was avoided. An additional series of six xenografts was analyzed for CK activity.

In Vitro Cell Cultures. Similar deproteinated extracts were made from cell cultures of 54A and 54B and analyzed for their metabolite content in order to compare the findings from xenografts with those of stroma-free cell cultures of the same tumors. Triplicate cultures of 3–5 $\times 10^7$ cells were examined for the content of ATP, PCr, and DNA. The concentrations were calculated in mol per cell.

In a separate experiment, the CK activity was determined (also in triplicate) in specifically prepared extracts of cell cultures of 54A and 54B.

Statistical Analysis. Comparison of the metabolic ratios and concentrations in xenografts 54A *versus* 54B, and in early *versus* late stage of growth, was done by the two-sided Wilcoxon test (Mann-Whitney test) and also, when the Gaussian distribution criteria were met, by parametric analysis (*t* test). In the latter case, the possibility of significant differences between variances was excluded by an *F* test (10). In the analysis of differences in the cellular ATP content *in vitro*, a *t* test was applied.

In Vivo ^{31}P-MRS. During measurements, the mice were restrained in a thin elastic polyvinyl chloride tube with the tumor protruding through a hole concentric with the radiofrequency coil. For installation, the mice were briefly anesthetized for 4–6 min with propanidid i.p. (Sombrevin, 500 mg/kg; Gideon Richter, Budapest, Hungary). Thus, during spectral recording, the mice were unanesthetized. A special probe with a 10-mm two-turn surface coil located over the tumor was used. Two different magnets were used: initially, a Bruker AM 250 spectrometer with a field strength of 6.5 Tesla applying 500 scans obtained at 2-s intervals (4) and, at a later stage, a Vivospec spectrometer (Otsuka Electronics, Inc.) with a wide-bore 2.9 Tesla magnet (Magnex, Inc.). In the latter case, a sufficient quality of the spectra was gained with 256 scans obtained at 2-s intervals. There was no selective enhancement of P_i or any other significant spectral changes by increasing the repetition time to 5 s. In the two magnets, the pulse width applied was 15 μs and 30 μs, respectively, corresponding to a flip angle of about 70°. In the spectra, the ATP/P_i ratio was calculated from the peak heights of β-ATP and P_i, since the line widths of the two peaks were identical and did not change during the experiment. This estimate has been validated and found safe as previously described in an identical setup (4) and by others (11). There was no systematic difference in ATP/P_i ratio obtained with the two different spectrometers. In animals restrained in the magnets, the spectra remained constant for at least two to three consecutive observation periods.

Contamination from underlying murine tissue was minimal in the setup, since the coil diameter was equal to or less than the tumor diameter. To ensure this, a few (<5%) extremely undersized tumors were excluded. The lack of murine contamination was confirmed by the observation that the ATP/PCr ratio in the tumor extracts was not different from that of the spectra.

Preparation and Analysis of Solid Tumor Extracts. In order to obtain *in vivo* concentrations of metabolites in the samples, the tumors were freeze clamped *in situ* and detached from skin and other adherent tissues, but with intact vessels, by application of a special procedure (4). The subsequent weighing, perchloric acid extraction, and neutralization to pH 7.00 were performed according to procedures described elsewhere (12). ATP, PCr, AMP, and ADP were measured by enzymatic assays (13), and P_i was measured by a colorimetric assay (14). The concentration unit applied is millimol per kg of tissue (mM).

The activity of creatine kinase (mU/mg tissue) was determined in extracts of each tumor type by a previously described assay (15). These extracts were prepared by homogenizing 1 volume tissue in 20 volumes ice-cold buffer (150 mM KCl, 15 mM $NaHCO_3$, 4 mM EDTA sodium, and 2 mM dithiothreitol; pH = 7.0). The homogenate was sonicated for 15 s at 30 W and centrifuged at 16,000 rpm for 20 min at 4°C.

Preparation and Analysis of Tumor Cell Extracts. Approximately 5 $\times 10^7$ cells/flask were harvested when the cultures were subconfluent. The medium was removed, and a defined volume of ice-cold 0.3 M trichloroacetic acid was added. After 5 min of mechanical agitation on ice, the cell homogenate was sonicated for 20 s at 30 W. Aliquots were taken for fluorometric DNA analysis (16), and the homogenate was centrifuged at 0°C at 5000 rpm for 15 min. The supernatant was neutralized with 0.6 M KOH, frozen in liquid nitrogen, and stored at −80°C.

PCr and ATP were measured in the extracts using the same assays as those used for analysis of the solid tumor extracts.

Results were expressed in μmol per mg DNA. The exact number of cells was unknown, so in order to obtain cellular concentrations, the ATP/DNA was subsequently adjusted for the different cellular (G_1) DNA content of 54A and 54B using the DNA index of 54A and 54B (8). The adjusted ATP/DNA fraction provided a relative measure of the cellular ATP content in the two cell lines. When multiplied with the amount of DNA per human cell, 5 pg (17), the fraction becomes a measure of the ATP concentration per tumor cell.

The cellular creatine kinase activity was determined in extracts prepared and analyzed as in the solid tumor extracts.

RESULTS

^{31}P-MRS. A gradual decrease in the ATP/P_i ratio was observed in the 12 tumors that were analyzed at weekly intervals during untreated growth. At the age of 25–35 days after transplantation, the tumor size was found to be optimal with regard to the coil diameter and the probe. Therefore, in the subsequent experiments comparing 54A and 54B, the tumors were analyzed at this stage of growth. As shown in Table 1, the ATP/P_i ratio found by ^{31}P-MRS was significantly higher in 54A tumors than in 54B tumors.

Solid Tumor Extracts. The tumor extract analysis was in accordance with the observation by ^{31}P-MRS of a decrease in the ATP/P_i ratio during growth. There was a significant difference in the ATP concentration and the ATP/P_i ratio between tumors sampled at days 25–35 and those sampled at day 60 after transplantation (Table 2). There was no significant variation over time in P_i or PCr (data not shown). All tumors were weighed following the freeze-clamping, and the data were analyzed for any correlation between tumor weight and absolute metabolite content or ratios. The significant change in ATP with time ("age") after transplantation (Table 2) reflects a correlation between the size of the individual tumor and metabolic status. There was, on the other hand, no general correlation between tumor size and the size of ATP/P_i or the concentrations of ATP, P_i, and PCr (data not shown).

The results of the biochemical analysis of the tumor extracts are summarized in Tables 3 and 4. No significant differences in P_i, ADP, AMP, or PCr were found (Table 3), whereas a statistically significant difference in ATP/P_i ratio between solid tumor extracts of 54A and 54B types was observed (Table 4). This difference was caused by a higher ATP level in 54A than in 54B. The phosphorylation potential, defined as the equilibrium constant of the ATP $\rightleftarrows$ ADP + P_i reaction, was significantly greater in extracts from 54A than from 54B tumors, and so was the adenine nucleotide sum (Table 4).

In both tumor lines, the PCr/ATP ratio was generally less than 0.5 (data not shown).

The CK activity in the solid tumor extracts was measurable, but very low. The CK activity was 2-fold higher in 54B tumors than in 54A tumors. The values were in the range of 1–3 units/g, which corresponds to less than 1% of that of muscle.

In Vitro Cell Extracts. The results of the ATP determinations in the *in vitro* cell cultures are given in Table 5. The six flasks

Table 1 *ATP/P_i ratio determined by ^{31}P-MRS of 54A and 54B xenografts*[a]

Xenograft	No.	ATP/P_i
54A	24	
Median [range]		0.90 [0.43–1.50]
Mean (SD)		0.86 (0.31)
54B	23	
Median [range]		0.64 [0.51–1.30]
Mean (SD)		0.69 (0.24)

[a] Test: nonparametric: $P < 0.05$ (Mann-Whitney); parametric: $P < 0.05$ (*t* test).

Table 2 *Median ATP/P_i ratios determined by chemical analysis of 54A and 54B xenografts*

Tumors were analyzed 25–35 days (early) or 60 days (late) after transplantation. The "early" values are also included in Tables 3 and 4.

	ATP/P_i	ATP (mM)	P_i (mM)
54A			
Early	0.38[a]	1.52[a]	4.10[b]
Late	0.26[a]	1.02[a]	3.85[b]
54B			
Early	0.23[a]	1.03[a]	4.06[b]
Late	0.15[a]	0.77[a]	4.40[b]

[a] Statistically significant difference between early and late values, $P < 0.05$, Mann-Whitney.
[b] Not significant.

were processed simultaneously and blinded with regard to cell type. In the analysis of data, the variance within the same cell type was considered a measure of the accuracy of the overall procedure, and therefore identical in the two groups. This allowed for the use of a parametric test (*t* test), which clearly demonstrated a significantly higher ATP content per cell in 54A than in 54B cultures. The P_i content (data not shown) was identical but very high in both cell types, because of interference with the phosphate content of the medium. In none of the cultures were measurable amounts of PCr found, and there was also no detectable CK activity in the cell cultures.

DISCUSSION

A decrease in the ^{31}P-MRS-determined ATP/P_i ratio with time during untreated growth was found in both SCLC tumor lines; this is in agreement with the findings in various other tumors and tumor transplants (1–3). The comparison of solid tumor extracts from day 30 and day 60 after transplantation strongly suggests that the decrease in ATP/P_i was caused by a decrease in ATP, whereas P_i remained constant. In contrast to the tumor age, there was no independent correlation between tumor size and ATP/P_i ratio in the tumor population as a whole. The absolute tumor size is essentially determined by host factors, vascular and nutritional, whereas the Gompertzian growth of tumors is a function of time. The decrease in ATP concentration with time may reflect the increase in the necrotic fraction during growth of a tumor by a dilution mechanism or reflect an overall decrease in the supply of oxygen and nutrients, which in return may play a causative role with regard to increased necrosis.

PCr was detected in small, but distinct, amounts in the xenografts, both by ^{31}P-MRS and by analysis of tumor extracts. Since the ATP/PCr ratio in the tumor extracts was similar to that found by ^{31}P-MRS of the tumors *in situ*, murine muscle or skin was not the source of ^{31}P-MRS-detectable PCr, as was reported in another model, in which the tumor spectra were influenced by the PCr content of skin muscle (18).

In the extracts of cell cultures, on the other hand, we found no PCr.

In a study applying high resolution ^{31}P-MRS in cell culture extracts of SCLC and non-SCLC lines, PCr was found in some,

Table 3 *Metabolite levels in xenografts 54A and 54B determined by chemical assays of extracts of solid tumors*

	No.	ATP (mM)	P_i (mM)	ADP (mM)	AMP (mM)	PCr (mM)	Weight (g)
54A	29						
Median [range]		1.52 [0.98–2.47]	4.10 [2.5–6.5]	0.36 [0.08–1.04]	0.053 [0–0.18]	0.53 [0.09–1.11]	0.23 [0.04–1.28]
Mean (SD)		1.51 (0.31)	4.18 (0.69)	0.36 (0.18)	0.059 (0.04)	0.63 (0.45)	
54B	21						
Median [range]		1.03 [0.72–1.35]	4.06 [2.6–6.1]	0.33 [0.20–1.04]	0.096 [0–0.30]	0.38 [0.04–0.96]	0.28 [0.05–2.68]
Mean		0.99 (0.249)	4.14 (0.76)	0.38 (0.18)	0.089 (0.077)	0.43 (0.19)	
Test							
Nonparametric		$P < 0.05$	ND[a]	ND	NS[b]	NS	NS
Parametric		$P < 0.001$	ND	ND	NS	—[c]	—

[a] ND, test not done, obviously no difference.
[b] NS, test performed, not significant.
[c] —, test not performed, distribution unfit for test.

Table 4 *Metabolic ratios in the solid tumor extracts from table 3*

	No.	ATP/P_i	Phosphorylation potential[a] (mol^{-1})	Adenine nucleotide sum[b] (mM)
54A	29			
Median [range]		0.38 [0.20–0.45]	1020 [190–510]	2.00 [1.24–2.63]
Mean (SD)		0.37 (0.05)	1420 (1110)	1.94 (0.35)
54B	21			
Median [range]		0.23 [0.11–0.33]	740 [180–1360]	1.54 [0.89–1.62]
Mean (SD)		0.24 (0.05)	780 (300)	1.51 (0.27)
Test				
Nonparametric		$P < 0.05$	$P < 0.005$	$P < 0.005$
Parametric		$P < 0.05$	—[c]	$P < 0.01$

[a] ATP/[ADP × P_i].
[b] ATP + ADP + AMP.
[c] Test not performed, distribution unfit for test.

Table 5 *Cellular ATP content determined in extracts of in vitro cell cultures of 54A and 54B*

Cell type	Flask no.	ATP/DNA[a] (mmol/g)	ATP/cell[b] (mol)
54A	1	1.23[c]	
	2	1.07[c]	5.0×10^{-15}
	3	0.72[c]	
54B	1	0.41[c]	
	2	0.39[c]	2.0×10^{-15}
	3	0.39[c]	

[a] Adjusted for difference in cellular DNA content by the DNA index: 2.5 (54A) and 1.3 (54B) (7).
[b] Mean values of ATP/DNA multiplied by DNA per cell (5 pg).
[c] Stastistically significant difference between cell types, $P < 0.01$, t test.

but not all SCLC lines (19). Based on the presence of immunodetectable creatine kinase BB, which is frequently observed in SCLC, the authors suggested that PCr was not seen because it was consumed by the creatine kinase pathway to maintain the ATP levels during preparation of cells. This explanation of the lack of PCr could not have been the case with the two tumor lines in the present study, since there was no measurable CK activity in the *in vitro* cell extracts and a measurable, but very low, CK activity (<1% of normal muscle) in the solid tumor extracts. Hence, our findings indicate that the PCr content of the solid tumors, detected by *in vivo* ^{31}P-MRS and biochemical analysis, originated from the stromal components and not from the tumor cells. The same conclusion holds for the CK activity.

By each of the three methods applied, a significantly higher ATP/P_i ratio was seen in the 54A subpopulation compared with the 54B tumors. The analysis of the solid tumor extracts revealed that this difference was due to a significantly higher ATP concentration in 54A than in 54B, whereas the P_i concentration was similar in the two tumor populations. This finding corroborates our previous observations in 54A and 54B tumors (4). There is good evidence that blood flow and tissue oxygenation correlate with the ATP/P_i in normal tissues as well as in tumor transplants (20–22). Accordingly, it was hypothesized that the ATP/P_i difference in the solid tumors of 54A and 54B type was due to extracellular (*e.g.*, vascular) factors. This explanation now seems ruled out by the present observation that 54A and 54B maintain their different ATP content *in vitro*. We conclude that the different ATP/P_i ratio and ATP concentration in solid tumors as well as in cell cultures of 54A and 54B reflect a true metabolic difference at the cellular level. The adjusted ATP/DNA fraction (Table 5) expresses the ATP content in 54A and 54B cells in G_1 phase. The ATP/cell calculation, however, represents an underestimate since, in tumors, not all of the cells are in G_1 phase. The small fraction of cells in cycle phases other than G_1 contributes with a greater-than-diploid amount of DNA. Thus, the average DNA content per tumor cell is slightly higher than the amount indicated by the DNA index, which is derived from the DNA content of G_1 fractions (9). This bias does not explain the great difference between 54A and 54B, since their cell cycle distribution is almost identical (8), but it might play an important role in interpreting smaller differences in cellular metabolite levels of different tumor cell lines and when comparing conditions with different cell cycle distribution (as *e.g.*, *in vitro* cells *versus* xenografted tumors). The calculated ATP concentrations ranged from 2.0×10^{-15} to 5.0×10^{-15} mol/cell (Table 5), which is in concordance with the reported ATP concentration in Ehrlich ascites cells of 3.6×10^{-15} mol/cell (23).

The higher ATP level in 54A tumors and cultured 54A cells could be interpreted in the light of the significantly different ATP/[ADP × P_i] equilibrium (Table 4), as an indicator of higher energy status in 54A than in 54B. Furthermore, the total adenine nucleotide concentration, ATP + ADP + AMP, which is usually fixed within narrow limits (24), was significantly higher in 54A tissue compared with 54B (Table 4). These discrepancies and the statistically significant difference in ATP content between two subpopulations of the same original SCLC tumor, in solid tumors as well as in cell cultures, indicate differences in the synthesis and/or utilization of ATP in 54A and 54B cells, thus implying a different metabolic phenotype of the two cellular subpopulations.

The metabolic response to irradiation of 54A and the less sensitive 54B was recently shown to be different (4). These findings suggested an association between steady-state ATP/P_i levels and radiosensitivity. Since the ATP/P_i ratio is sensitive to changes in the oxygen supply, and radiosensitivity is related to the presence of oxygen, a major role of oxygen in the maintenance of the differences between 54A and 54B was plausible. In the present study, however, the metabolic differences in solid tumors of 54A and 54B types were also demonstrable in the same cells *in vitro*, which makes variations in the oxygen supply an unlikely explanation of the different ATP levels. Accordingly, factors other than the presence of oxygen may govern the difference in radiosensitivity.

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