Phosphorous metabolites and steady-state energetics of transformed fibroblasts during three-dimensional growth

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Kunz-Schughart, Leoni A., and James P. Freyer. Phosphorous metabolites and steady-state energetics of transformed fibroblasts during three-dimensional growth. Am J Physiol Cell Physiol 283: C1287–C1297, 2002; 10.1152/ajpcell.00097.2002.—Rat1-T1 and MR1 spheroids represent separate transformed phenotypes originated from the same rat fibroblasts that differ in three-dimensional (3D) growth kinetics, histological structure, and oxygenation status. In the present study, 31P-NMR spectroscopy of perfused spheroid suspensions was used to investigate cellular energetics relative to 3D growth, development of necrosis, and cell cycle distribution. Both spheroid types were characterized by a remarkably low amount of free (inorganic) phosphate (Pi) and a low phosphocreatine peak. The ratio of nucleotide triphosphate (NTP) to P, ranged between 1.5 and 2.0. Intracellular pH, NTP-to-P, ratio, and NTP/cell remained constant throughout spheroid growth, being unaffected by the emergence of oxygen deficiency, cell quiescence, and necrosis. However, a 50% decrease in the ratio of the lipid precursors phosphorylcholine and phosphorylethanolamine (PC/PE) was observed with increasing spheroid size and was correlated with an increased G1/G0 phase cell fraction. In addition, the ratio of the phospholipid degradation products glycerophosphorylcholine and glycerophosphorylethanolamine (GPC/GPE) increased with spheroid diameter in Rat1-T1 aggregates. We conclude that changes in phospholipid metabolism, rather than alterations in energy-rich phosphates, reflect cell quiescence in spheroid cultures, because cells in the inner oxygen-deficient zones seem to adapt their energy metabolism to the environmental conditions before necrotic cell destruction.

energy metabolism; tumor biology; nuclear magnetic resonance spectroscopy; phospholipids; quiescence

MAGNETIC RESONANCE SPECTROSCOPY (MRS) is increasingly utilized as an in vivo method to monitor cell metabolism and tissue oxygenation in various organs and diseases (17, 27, 41, 49). New approaches include its application in neuropsychiatric and toxicity research (5, 10, 46, 61), and it is widely applied in pharmacokinetic and comparative physiological studies (47, 50, 56, 67, 84). MRS has also become a powerful technological tool providing noninvasive access to tumor bioenergetic state because of its most attractive feature of nondestructively measuring chemical compounds in intact, living tissues (17, 27). Combinations of advanced MRS techniques with labeled compounds and genetic manipulation are now allowing in situ measurements of specific metabolic pathways in tumors (8, 58, 67, 71, 87).

Experimental, preclinical, and clinical spectroscopy results indicate that cancers have typical metabolic characteristics that might be of diagnostic and/or prognostic relevance and could be used to some extent as predictors of cancer treatment outcome (17, 24, 31, 42, 45, 51, 54, 57, 64, 70). Among other things, measurement of tumor energetics by 31P-NMR spectroscopy provides useful information about tumor oxygenation/hypoxia and potential therapeutic resistance. However, there is some controversy about the relevance of the additional parameters that can be monitored in tumors via phosphorous NMR, e.g., phospholipid metabolism (15, 16, 26, 43, 44, 62, 68, 79). It was previously reported that most human tumors contain high concentrations of phosphomonoesters, with the major compounds being identified as phosphorylethanolamine (PE) and phosphorylcholine (PC) (15, 54, 72). Yet most of these studies raise an important question: Are modifications in the phospholipid metabolism of tumor cells due to an increase in proliferative activity rather than to tumorigenic conversion?

Over the past decade much work has also concentrated on establishing correlations between 31P-NMR data and physiological or environmental factors to analyze more precisely cellular mechanisms influencing the NMR spectra. Parameters of energy status that have been used in 31P-spectroscopy are typically the ratio of nucleotide triphosphate (NTP) and phosphocreatine (PCr) to inorganic phosphate (Pi) and related ratios as well as the intracellular pH (pHi). Although changes in these parameters have been correlated with necrosis and tumor oxygen status in some particular tumors, no correlation was found in others (17, 24, 25, 27, 42, 48, 58, 59, 60, 74, 85).

The interpretation of in vivo tumor NMR data to address basic metabolic questions is hampered by several factors, e.g., tissue/tumor heterogeneity, in partic-
ular tumor vascularization and nutrient supply, and lack of experimental control over environmental conditions (34, 48, 73). Multicellular spheroids represent a well-established in vitro model for investigation of the interrelationship among growth/proliferation kinetics, viability, and energetic state of tumor cells (23, 39, 53). An NMR system for viably maintaining a stirred suspension of multicellular spheroids during NMR spectroscopy has been established in our laboratory (20). Based on this technical development, a few 31P-NMR studies have focused on the phenomenon of the development of a quiescent cell population in the inner viable cell rim of spheroids and the emergence of necrosis. For EMT6/Ro mouse mammary carcinoma spheroids, a negative correlation between the ratio of the membrane phospholipid precursors PE and PC and the S phase cell fraction was documented whereas pH\textsubscript{i} and the ratios of NTP to P\textsubscript{i} and PCr to P\textsubscript{i} did not significantly change as a function of spheroid size, proliferative activity, and/or extent of central necrosis (21).

To investigate in more detail the relationship among proliferation, oxygen supply, cell viability, and cellular energetics we have studied two established and well-characterized oncogene-transfected rat embryo fibroblast spheroid types using 31P-MRS. Although these two cell lines are genetically closely related, thus representing to some extent cellular heterogeneity in a rat tumor, they clearly differ in three-dimensional (3D) culture in the following characteristics: 1) growth kinetics, proliferative activity and cell viability; 2) cell shape and histological structure involving the development of necrosis; 3) oxygen consumption and distribution, 4) mitochondrial activity, and 5) ATP, glucose, and lactate concentrations as measured by various bioluminescence techniques (35–38, 40). In the present study, we report 31P-NMR spectroscopic data on these two spheroid types as a function of spheroid size, cell cycle distribution, and the development of necrosis.

## MATERIALS AND METHODS

### Cell lines and spheroid culturing.

The highly tumorigenic cell clones Rat1-T1 and MR1 were derived from two different immortalized rat fibroblast cell lines by T24Ha-ras transfection. The parental cell lines (Rat1 and MR1) were originated from a mouse plasmacytoma. Ras transfection was carried out with the oncogene plasmid \( \text{pH06T1} \), which contains a single-base mutant 6.6-kb \( c-Ha-ras \) gene isolated from a human bladder carcinoma. Cell lines, oncogenes, and the transfection protocol have been described in previous publications (35–38, 40).

Monolayer stock cultures were routinely subcultured and periodically tested for mycoplasma contamination via standardized techniques as described previously (36). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Gaithersburg, MD) with the addition of 10% fetal calf serum (Sigma), 100 IU/ml penicillin, and 100 \( \mu \)g/ml streptomycin sulfate (GIBCO).

Spheroids were initiated and cultured essentially as documented previously (37). Spheroid growth was initiated either in microbiological petri dishes or 0.5% (wt/vol) agar-coated culture dishes (1–2 \times 10^6 cells per 100-mm dish), and spheroids were maintained in spinner flasks with continuous stirring after an initiation interval of 4 days. Complete DMEM as described above was used for spheroid culturing. Medium was replenished daily, and the number of spheroids was continuously reduced to keep the cell count in the spinner flask relatively constant, ensuring an essentially constant medium condition throughout growth. Monolayer and spheroid cultures were kept in a humidified atmosphere with 5% (vol/vol) CO\(_2\) in air at 37°C.

### Spheroid volume and histology.

The mean (±SD) spheroid volume within hand-selected spheroid populations was calculated from 50 individual spheroid diameter values. For each spheroid observed, minor and major diameters were measured and averaged with a calibrated image processing apparatus consisting of an inverted microscope (Stemi SVI; Zeiss) connected to a solid-state camera (COHU Electronics) with monitor and a Macintosh-based computer system for image analysis (NIH Image).

For histological observation (e.g., thickness of the viable cell rim), spheroids were fixed in 2.5% (vol/vol) glutaraldehyde or 10% buffered formalin, mounted in paraffin, serially sectioned into 5-μm-thick slices, and stained with hematoxylin and eosin. Central sections were detected taking into account an average shrinkage of 18% due to histological processing. Measurements of the thickness of the viable cell rim in central sections were done with a calibrated reticle in a microscope as detailed previously (23).

### Cell counting, volume, and cell cycle distribution.

Cells dissociated from spheroids by mild trypsinization (0.125–0.25% trypsin) and mechanical means were counted with a standard particle counter (Coulter, Hialeah, FL) equipped with a pulse height analyzer (Nucleus). Acellular debris was excluded from the cell count by determining a region of interest for the cell volume as described previously (20). Cell volume distributions of ≥10,000 cells per cell population were used to calculate the average cellular volume based on the calibration of the system with polystyrene microspheres.

For DNA content analysis, cells were prepared for flow cytometric assay via a routine mithramycin staining technique as described previously (18). A flow cytometer custom built at Los Alamos National Laboratory (30) with laser excitation wavelength of 457 nm and emission wavelengths >512 nm was used to analyze stained single-cell suspensions. DNA histograms containing 2.5 × 10^6 cells were collected, and cell cycle distributions were calculated with the MultiCycle AV program (Phoenix Flow Systems, San Diego, CA).

### 31P-NMR measurements.

For NMR experiments, hand-selected groups of spheroids were transferred into a specially designed perfusion chamber connected to a perfusion system as documented previously (21). We have devised a novel type and size of perfusion chamber for maintaining a viable suspension of multicellular spheroids during NMR spectroscopy, which increased the temporal resolution by a factor of 10 compared with previous studies (Fig. 1A). By using completely relaxed acquisition conditions, a 1-h spectrum with a signal-to-noise ratio of ≥10 could be collected using only 2–3 × 10^6 cells. Other advantages of the new type of chamber include the integrated containers for standard solution that can be easily filled from the outside and the fact that the cell suspension is mixed by the perfusion flow, avoiding the use of a stirring device controlled by an external motor.

Temperature, P\textsubscript{O2}, and extracellular pH in the chamber were monitored continuously during one experimental series as described previously (20). Measurements were carried out at 37 ± 0.5°C, 20% O\(_2\), and a pH of 7.3 ± 0.1 in supplemented
RESULTS

It was documented previously that spheroid volume growth and histology of the two oncogene-transfected fibroblast cell lines differ, with MR1 aggregates reaching maximum volumes at days 12–14 and Rat1-T1 spheroids entering spheroid plateau phase approximately at days 20–24 after an initiation interval of 4 days in petri dishes or agar-coated culture dishes (36). This growth behavior has been confirmed in the present study (Fig. 2A), and cell cycle distributions have been determined via flow cytometry in parallel (Fig. 2, B–D). Spheroid volume growth was mathematically described by the Gompertz equation as detailed previously (36). The corresponding fits are shown as lines in Fig. 2A. For cell cycle distributions we could show that the number of cells in G1/G0 significantly increased as a function of the spheroid size (P < 0.01) and the number of cells in S phase was continuously reduced, with minimum values of 10–15% of the total. At the same time, the proportion of cells in G2/M phase significantly decreased in Rat1-T1 but not MR1 spheroids, suggesting a G2/M phase block in these cells as well. In general, cell growth kinetics were reflected by the cell cycle distributions, with 1) a lower proportion of cells in S phase within the slower-growing Rat1-T1 as opposed to MR1 spheroids and 2) a clear reduction in the S phase cell fraction and an increase in the percentage of cells in G2/M phase accompanying spheroid volume growth retardation in both spheroid types.

Cell count per spheroid, cell volume, and thickness of the viable cell rim recorded in the present study correspond with data published previously (36). Within the spheroid size range investigated via NMR spectroscopy, the cell number per spheroid exponentially in-

31P-NMR Analysis of Rat Fibroblast Spheroids

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phosphate-free medium. The volume of the perfusion chamber inside the NMR coil sensing region was 0.8–1 ml with a total chamber volume of ~6 ml. The flow rate varied between 2.4 and 3.4 ml/min depending on spheroid size. Under these conditions, spheroid growth in the chamber was comparable to that in the spinner flask culture, and pH_i, P_i level, as well as NTP-to-P_i ratio were constant for several hours (Fig. 1B).

31P-NMR analysis. Phosphorous NMR spectra were recorded at 161.97 MHz with a Bruker AM400wb spectrometer system (9.7 T). A 10,000-Hz (61.74 ppm) sweep was accumulated in 4,096 data points in an acquisition time of 0.27 s with a recycle time of 10 s after a 90° pulse. Spectra were stored every 360 scans (60 min 47 s), and 5–20 spectra were added for subsequent peak analysis. Chemical shifts were recorded with respect to 85% H₃PO₄ and were analyzed by setting the resonance of the external methylenediphosphonic acid (MDA) standard to 16.8 ppm. Peak height analysis was carried out with Bruker analysis software (Bruker Instruments, Billerica, MA). Peak heights in a spectrum within a given experiment were referenced to the corresponding height of the MDA peak (25 mM; 16.4 µl) in the spectrum (20, 21).

Statistical analysis. Pairwise comparisons were performed with a two-tailed Student’s t-test based on the means and standard deviations of three to five repeated measurements, with differences considered statistically significant when P < 0.05. Correlations between two parameters were determined by least-squares best fits to a linear equation, and the parameters were considered significantly correlated when r > 0.5.

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Fig. 1. A: schematic diagram of a novel type of perfusion chamber (10 mm in diameter) for maintaining viable suspensions of multicellular spheroids during NMR spectroscopy. The perfusion chamber was equipped with an integrated container for standard solutions, a temperature sensor, a medium input with the perfusion stream mixing the spheroid suspension, a medium output (1), and an overflow medium output (2). The sensing volume of this chamber was ~600 µl as indicated by the dimension specifications. B: analysis of sequential 1-h spectra of a suspension of Rat1-T1 spheroids over an experimental period of 14 h. Points represent individual determinations of intracellular pH (pH_i), γ-nucleoside triphosphate (NTP), and γ-NTP-to-inorganic phosphate (P_i) ratio with solid lines showing linear least-squares best fits to the data.
creased for both spheroid types and the average cell volume remained relatively constant. The mean thickness of viable cell rim was 202 ± 26 μm in Rat1-T1 (n = 26), whereas the viable cell rim of MR1 spheroids was slightly smaller than described previously, measuring some 279 μm with a standard deviation of 44 μm (n = 37). This value reflects the high variance in the thickness of viable cell layers within this spheroid type, probably due to its loose cell packing density associated with a high rate of cell shedding on the spheroid surface. Although Groebe and Mueller-Klieser (29) showed that necrosis expands quickly after initial induction, over the spheroid size range investigated in this study there was no significant change in the thickness of the viable cell rim with increasing spheroid diameter, as has been reported for other cell lines (23).

Because the energetic parameters analyzed via 31P-NMR spectroscopy were constant over an experimental period of >15 h in the new type of perfusion chamber, we have added at least four 1-h spectra for subsequent further analysis. Figure 3 shows representative added 31P-NMR spectra of small and large Rat1-T1 and MR1 spheroids. Figure 4 summarizes the results on the cell energetic parameters pH, γ-NTP/Pi, and γ-NTP per cell quantified from the added NMR spectra. It could be shown that neither pH nor the γ-NTP-to-Pi ratio significantly changed throughout spheroid growth (r < 0.1). Also, the development of a histologically detectable central necrotic region at spheroid sizes of 500–600 μm for Rat1-T1 and 800–900 μm for MR1 aggregates was not accompanied by systematic changes in pH and γ-NTP/Pi. However, cell line-specific differences of the two tumorigenic fibroblast spheroid types are not only manifested in spheroid proliferation, structure, and oxygenation but could also be identified in the NMR spectra. As a result, MR1 spheroids were characterized not only by a higher proliferative activity and a higher S phase cell fraction during spheroid volume growth but also by a 1.5-times lower overall γ-NTP-to-Pi ratio compared with Rat1-T1 spheroids. In addition, a slight but significant (r = 0.91) decline in the γ-NTP concentration/cell as a function of spheroid diameter was observed for Rat1-T1 but not MR1 spheroids. Because the cell volume in both MR1 and Rat1-T1 spheroids did not consistently change as a function of spheroid size, the negative correlation between γ-NTP per Rat1-T1 cell and spheroid diameter was not due to cell volume alterations.

Analysis of phosphomonoesters and phosphodiesters from the NMR spectra showed that the PC-to-PE ratio systematically decreased with spheroid diameter, which might be associated with cell quiescence (Fig. 5C). As documented in Fig. 5, A and B, the modifications in PC/PE were due to a significant reduction in PC with spheroid growth (r > 0.53) while the PE level per cell was constant throughout spheroid growth (r < 0.1). In addition, MR1 spheroids that were characterized by a shorter spheroid volume doubling time and a higher proportion of S phase cells accompanied by a lower

Fig. 2. A: spheroid volume/size as a function of time in spinner flask culture after an initiation interval of 4 days in agar-coated culture dishes. Proportion of cells in S phase (B), G2/M phase (C), and G1/G0 phase (D) is shown as a function of the spheroid diameter determined in dissociated spheroid cell suspensions via flow cytometric DNA analysis. ○, Rat1-T1; •, MR1. Lines show least-squares best fits to the Gompertz equation in A and linear least-squares best fits to the data points in B–D.
G1/G0 phase cell fraction exhibited higher PC/PE compared with Rat1-T1 aggregates of the same size category. Because the decline in PC was not significantly different in Rat1-T1 and MR1 spheroids as a function of spheroid diameter \( (P > 0.05 \text{ comparing the slopes}) \), differences in PC/PE between the two cell lines seem to relate to the increased amount of PE in the Rat1-T1 spheroids.

In contrast to the phospholipid precursor PC, no significant correlation of the single-phospholipid degradation products GPC and GPE and spheroid size was found \( (r < 0.05, \text{ Fig. 6, A and B}) \), although the calculated ratio GPC/GPE was positively correlated with the aggregate size of Rat1-T1 cultures \( (P < 0.05) \) as shown in Fig. 6C. The increase in GPC/GPE throughout Rat1-T1 spheroid growth mirrors the changes in the phospholipid precursor ratio PC/PE. A similar correlation was not shown for MR1 cells in spheroids because the GPE peak was not consistently detectable in the added spectra (see, e.g., Fig. 3) and peak height analysis could not be performed.

The observations of this study can be summarized as follows. First, both fibroblast spheroid types were characterized by an extremely low PCr peak, which could be observed in the added but not the single 1-h spectra. Second, P; was only moderate relative to γ-NTP; thus γ-NTP/P; was clearly >1 for both spheroid types and did not significantly change throughout spheroid growth, similar to pH. Third, focusing on the phosphonoesters, the same characteristics were found in Rat1-T1 and MR1 spheroids, with a constant cellular PE level and a decrease in PC concentration per cell throughout growth resulting in a reduction of PC/PE as a function of spheroid size. Fourth, a slight increase in the phosphodiester GPC per cell could be recorded for both spheroid types whereas the cellular concentration of the phospholipid degradation product GPE was a constant function of spheroid size (Rat1-T1 spheroids) and GPC/GPE clearly increased throughout growth of Rat1-T1 spheroids.

DISCUSSION

Over the past 10 years, fibroblasts transformed to differing extents have gained experimental importance, leading to a deeper insight into mechanisms underlying the multistep process of transformation. Recently, four differently transformed rat fibroblast cell lines have been well characterized in multicellular spheroid culture (35, 36, 40). Among other things, it could be demonstrated that even fibroblast clones that were originated from the same stem cells and belonged to a similar transformation stage such as Rat1 and M1 cells or Rat1-T1 and MR1 cells showed a variety of pathophysiological differences in two-dimensional (2D) and, in particular, 3D culture. The two diploid cell lines used for the present investigation (Rat1-T1 and MR1) represent tumorigenic, fully transformed phenotypes within an oncogene-dependent two-step transformation model that differ only in the immortalization process. As documented previously, differences of Rat1-T1 and MR1 spheroids in vitro that might represent in vivo heterogeneity of tumor physiology and microenvironment include the following: 1) development of ne-
crosis, 2) thickness of viable cell rim, 3) proliferative activity, and 4) oxygen gradient (35, 36).
We have carried out phosphorous magnetic resonance measurements of perfused suspensions of spheroids to verify whether the differences in oxygen availability, cell viability, and proliferative activity in Rat1-T1 and MR1 spheroids are reflected by any of the steady-state energetic parameters that can be determined from $^{31}$P-NMR spectra. Investigations on the energy metabolism of these morphologically well-defined spheroid types provide useful information on tumor cell biology because changes in tumor energetics such as ATP level and pH have been positively correlated with oxygen supply, glucose distribution, and/or necrosis in some tumors in vivo and in some but not all experimental systems in vitro (17, 24, 25, 27, 42, 48, 58–60, 74, 85). One interpretation of some controversial data in the literature has been discussed by Steen (74), who suggested different types of hypoxia, with metabolic hypoxia resulting in mitochondrial impairment in cells associated with a drop in ATP and a radiobiological-type hypoxia resulting in attenuated cell death related to radiation-sensitive tissue levels of oxygen. As a result, metabolic hypoxia could be detected in the $^{31}$P-NMR spectra, whereas the radiobi-

![Fig. 4. pH (A), γ-NTP/Pi (B), and relative γ-NTP/cell values (C) as a function of the spheroid diameter determined in viable suspensions of 2 differently transformed rat fibroblast spheroid types with $^{31}$P-NMR spectroscopy. ●, Rat1-T1; ○, MR1. Lines show linear least squares best fits to the data. Error bars indicate the variation in spheroid size within each set of added spectra due to spheroid growth.](http://ajpcell.physiology.org/)

![Fig. 5. Relative phosphorylcholine (PC) level per cell (A), relative phosphorylethanolamine (PE) level per cell (B), and PC-to-PE ratio (C) as a function of the spheroid diameter determined in viable suspensions of 2 differently transformed rat fibroblast spheroid types with $^{31}$P-NMR spectroscopy. ●, Rat1-T1; ○, MR1. Lines show linear least-squares best fits to the data. Error bars indicate the variation in spheroid size within each set of added spectra due to spheroid growth.](http://ajpcell.physiology.org/)

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logical hypoxia would be invisible. Previous work with spheroids does not support this hypothesis (20, 21). However, in the spheroid types investigated previously, metabolic hypoxia was accompanied by necrotic cell death, which probably contributes nothing to the \(^{31}\)P spectrum (27, 81). With the MR1 spheroid type, which contains a 10–15% fraction of viable cells under metabolically hypoxic conditions, we have an excellent model to verify whether metabolic hypoxia is reflected by changes in the NTP resonances. In addition, MR1 and Rat1-T1 spheroids are characterized by a decrease in mitochondrial activity both as a function of location in the spheroid and throughout growth (37–39), as has been reported for other cell lines (18, 19).

As opposed to phosphorous spectra determined for EMT6 (mouse mammary carcinoma) and 9L (rat gliosarcoma) spheroids, both tumorigenic fibroblast aggregate types were characterized by a remarkably low amount of free phosphate (P\(_i\)) whereas no PCr could be observed. Consequently, the ratio of NTP to P\(_i\) ranged between 1.5 and 2.0 in Rat1-T1 and MR1 spheroids compared with a value of ~1.0 for EMT6 and 9L spheroids (20, 21), suggesting large differences in the metabolic turnover rates of tumorigenic rat fibroblasts and these other tumor cells in 3D culture. However, for all spheroid types tested so far, including Rat1-T1 and MR1, the NTP-to-P\(_i\) ratio and pH\(_i\) remained constant as a function of the spheroid diameter. A significant decline in the NTP per cell throughout growth was shown only for Rat1-T1 spheroids and was confirmed by bioluminescence measurements of the ATP concentration (\(\mu\)mol/g) in the viable cell rim of this spheroid type (38). However, this decrease was positively correlated with reduction in cell volume and NTP/cell volume was constant.

The lack of correlation between pH\(_i\) or NTP/P\(_i\) and the development of necrosis and cell quiescence in both fibroblast spheroid types implies that tumor cells in the inner, nutrient-deficient spheroid areas adapted their energy metabolism to the changing microenvironment. Similar results obtained for other spheroid types indicate that the maintenance of a constant ratio of high-energy phosphates to P\(_i\) via cellular metabolic adaptation might be a general aspect of viable cells within 3D culture (21). It must be pointed out that the majority of MR1 spheroids with a diameter >1,000 \(\mu\)m consisted of an inner 50- to 75-\(\mu\)m-thick region of viable but cell cycle-arrested cells in which no oxygen could be detected with oxygen-sensitive microelectrodes. This zone corresponds to ~15% of the total spheroid cells, so that a large decrease in energy metabolism in this subpopulation of cells should have been detected as a decrease in the averaged values reported in this study. Even in this spheroid type, NTP/P\(_i\) was constant as a function of spheroid size and occurrence of the oxygen-deficient viable cell zone was not reflected by a drop in NTP/P\(_i\). We conclude that quiescent MR1 cells are highly resistant to lack of oxygen as opposed to other cell types such as Rat1-T1, EMT6, or 9L cells in 3D culture (21, 36) and that they may compensate for the resulting energy loss by utilizing additional, nonaerobic energetic sources such as glucose or glutamine. Preliminary experiments on glucose consumption per spheroid volume unit indicate that glucose is not the major factor involved in anaerobic energy compensation, because 1) there is no significant difference in glucose uptake of Rat1-T1 and MR1 spheroids and 2) glucose uptake decreases as a function of spheroid diameter (unpublished data), as has been shown for other cell types (22). Bourrat-Floeck and coworkers (9) described the phenomenon of lactate utilization in large EMT6 spheroids, whereas small spheroids produced large amounts of lactate as expected. It may be
speculated from their observation that a similar mechanism is activated in quiescent, oxygen-deficient MR1 cells. Determination of lactate and glucose levels and uptake/release rates in the two spheroid types with an advanced bioluminescence technique and routine enzymatic tests is in progress (86). The contribution of glutathione (GSH) in the regulation of energy metabolism as an antioxidant agent may also be considered, because Romero et al. (64) showed that GSH concentration per cell may change throughout spheroid growth, with a sharp increase around the diameter where necrosis occurs in V79 hamster lung cell spheroids. In summary, our data support previously reported work with spheroids and imply that cellular metabolic adaptation in the inner spheroid regions might be a general phenomenon in 3D cultures despite a changing microenvironment, nutrient deficiency, and loss in mitochondrial activity. The fact that mitochondrial activity does not affect NTP/Pi in the cells seems peculiar. However, Rasmussen et al. (63) showed that azide, an inhibitor of mitochondrial respiration, had no effect on the ATP concentration if adequate levels of glucose were present. Investigations by Teutsch et al. (78) using a modified Lowry technique for glucose detection indicate that sufficient glucose is available even in the center of large spheroid cultures.

Phospholipid metabolites have been discussed as indicators of cancer cell function, with two main kinds of phospholipid-related peaks that appear in the phosphorous spectrum: 1) phosphomonoesters PC and PE synthesized by the enzymatic activity of specific kinases that catalyze the first step of phospholipid biosynthesis in vivo and 2) phosphodiester GPC and GPE, the two major phospholipid breakdown products (68, 83). Data in the literature indicate that the relationship between tumor growth and progression, respectively, and single components in the phospholipid metabolism seems to be ambiguous (4, 7, 43, 44, 52, 55, 71). However, numerous recent studies have demonstrated a correlation between mitogenic stimulation and/or oncogenic transformation of cells and acute changes in phospholipid pathways such as transient increases in concentrations of certain metabolites and an increase in lipid turnover rate (1–3, 11, 13, 28, 33, 66, 82). In most cases, enhanced phospholipid biosynthesis and reduced phospholipid breakdown were observed in long-term chronic situations such as tumors and high phosphomonoester (PME) levels have been hypothesized to be the best biochemical marker to distinguish some cancerous from normal tissues (16, 17, 43, 52, 55, 62, 65) and to monitor tumor treatment outcome (69, 75). Some but not all of these observations might be due to the fact that under certain conditions PC strongly correlates with cell proliferation (4, 6, 21, 26, 62, 68, 72, 82). Ting et al. (80) compared the metabolism of different human breast cancer cells and normal mammary epithelial cells with a similar proliferative activity. They showed that, in contrast to high-energy phosphates and aerobic glycolysis, which did not reveal distinct differences between normal and cancer cells, levels of PC and PE were significantly higher in the tumor cells. In accordance with this finding, Cox et al. (12) demonstrated that liver tumors are characterized by an increase in PE and PC signals and a decrease in GPC and GPE signals, with the spectral changes being independent on tumor type, i.e., hepatocellular carcinoma, secondary adenocarcinoma, or squamous cell carcinoma. The higher levels of PE and PC in tumor extracts in vitro compared with normal tissue correlated with an increased PME peak seen in vivo. Lyng et al. (44) demonstrated that PME and PDE in 31P-NMR spectra in different human melanoma xenografts in vivo did not correlate with the rate of tumor cell proliferation and S phase cell fraction. In addition, Abrahà et al. (1) and Street and Koutcher (75) could not confirm a significant effect of different therapeutic modalities on phospholipid precursors and catabolites in either perfused RIF-1 fibrosarcoma cells or extracts of a murine breast carcinoma cell system. They concluded that phospholipid precursors and breakdown products are of limited value in prediction of tumor treatment outcome as suggested by others who found specific changes in particular in the phosphomonoester resonances associated with reduced cell viability and proliferation after radio- or chemotherapy (16, 42, 45, 51, 68, 74). Despite contradictory results with phospholipid measurements, increased total choline levels in tumors are being investigated as a means of detecting prostate cancer with 18F-labeled choline and positron emission spectroscopy (14).

In contrast to the lack of relation between the NTP signal and cell quiescence and necrosis, respectively, a clear negative correlation between PC/PE and the spheroid diameter could be shown with the faster growing/proliferating spheroid type MR1 characterized by a higher overall PC/PE. Analysis of the single parameters PC and PE per cell revealed that the alteration is solely due to a decrease in the PC peak whereas PE/cell remains constant throughout spheroid growth. Figure 7 documents the correlation between PC/PE

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**Fig. 7.** PC/PE determined in viable suspensions of 2 differently transformed rat fibroblast spheroid types with 31P-NMR spectroscopy as a function of the G1/G0 cell fraction analyzed from flow cytometric DNA histograms of dissociated spheroid cells. ●, Rat1-T1; ○, MR1. Line shows linear least-squares best fits to the entire data set (R = 0.711); dashed lines show linear least-squares best fits to the individual data sets (Rat1-T1, r = 0.766; MR1, r = 0.605).
and the proportion of cells in G1/G0 phase, which can be estimated from Fig. 2C. Linear regression analysis of PC/PE vs. G1/G0 phase cell fraction indicates that the correlation might be cell line independent for the fibroblast cell system tested. It must be kept in mind that the absolute amounts of PC and PE could vary between different cell lines, as is clearly shown for 2E (Fig. 5B). Thus, although the absolute value of PC/PE may not correlate with proliferation when comparing different cell lines, our results support the hypothesis of a general correlation between lipid (precursor) metabolism and proliferative status. However, from the analysis of our data we assume that the accumulation of cells in G1/G0 and probably the occurrence of G0 phase cells itself are reflected by a change in phospholipid metabolism rather than the decrease of actively cycling cells. Because it was proposed several years ago that an increased PC level might be a marker of cell differentiation (1, 2, 28), future investigations will have to determine whether the accumulation of G0 phase cells in tumors is similar to cell differentiation in normal tissue in terms of phospholipid metabolism.

It was also hypothesized in the early 1980s that an increase in the phospholipid breakdown products GPE and GPC may be a indicator of the necrotic fraction in tumors as a consequence of membrane degradation (27). However, Smith et al. (72, 73) cited some breast tumors that contained appreciable levels of GPE and GPC without gross necrosis. 3D cultures such as spheroids and rat tumor cells, Freyer et al. (21) found no correlation of the changes observed in phospholipid precursor and degradation products will require more sophisticated NMR approaches such as 13C-labeled precursors (32, 66) or NMR-visible analogs (76, 77).

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