Proliferative activity and tumorigenic conversion: impact on cellular metabolism in 3-D culture

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WITHIN THE PAST FIFTEEN YEARS, research on oncogenesis has become one of the most active areas of biological investigation. To date, the majority of this work has been molecular in focus, and little has been done to discover and understand pathophysiological variations of tumor vs. normal cell phenotypes that are relevant for the design of nonsurgical therapeutic treatments such as radio- and chemotherapy. Studies on tumor cell energetics, for example, have been a field of controversial discussion since Warburg et al. (45) hypothesized respiratory deficiency and elevated anaerobic glycolysis to be involved in carcinogenesis. Today, it is generally accepted that many but not all cancers are characterized by a highly deviated metabolism, and some modifications have been considered as diagnostic factors. However, it remains unclear whether these energetic alterations are primarily and directly due to the extent of transformation or rather accompany enhanced proliferative activity of these cells.

The biological significance and clinical relevance of tumor spheroids have been well documented in the literature and have been summarized in several comprehensive reviews (e.g., Refs. 23, 30, 31, 40). It is well accepted that spheroids mimic the specific environment of malignant cells in solid tumors more closely than conventional monolayers. This includes cell-cell and cell-matrix interactions, metabolic gradients, cellular viability, and differentiation. Mechanisms involved in gene regulation, such as cell cycle control or DNA repair, have been demonstrated to be very similar to those found in vivo and often different from those observed in adherent monolayers. From these findings it can be concluded that spheroids represent an appropriate model system for the registration of metabolic effects that are associated with malignant transformation. Multicellular spheroids have therefore been widely applied as an in vitro system to investigate the association of specific microenvironmental factors with success or failure of tumor therapy. Previous studies using a variety of tumor spheroids have shown that the oxygen uptake of tumor cells at advanced growth stages is considerably lower than that of rapidly dividing cells despite optimal oxygen availability (e.g., Refs. 8, 9, 15, 32). This indicates that oxygen consumption in three-dimensional (3-D) culture correlates with the proliferative activity supporting the hypothesis that oxygen metabolism rather reflects tumor cell growth characteristics than transformation state. However, tumorigenic
spheroid types have never been directly compared with their nontumorigenic ancestors, and conclusions on metabolic changes associated with either aggressive tumor growth and/or tumorigenic conversion of spheroid cells are rather ambiguous.

In an attempt to examine cellular metabolism of cells transformed to different extents in 3-D culture, we have previously established and characterized a myc/ras-dependent two-step carcinogenesis spheroid system (21). This system is based on Weinberg's generalized model for tumorigenic conversion of rodent cells, with the first step being immortalization, conferred by the activity of a nuclear oncoprotein, and with the second step being transformation, conferred by the activation of a cytoplasmic oncoprotein (25, 47). Cell types involved in this study were spontaneously immortalized Rat1 and myc-transfected M1 cells that are non- or poorly tumorigenic in vivo and highly aggressive ras-transformed Rat1-T1 and MR1 cells. The nontumorigenic cell types show poor 3-D aggregation and proliferation, whereas ras transfectants are characterized by anchorage independence, growth in semisolid media, spheroid formation in vitro, and rapid spheroid growth. In addition, spheroid types extremely differ in cell morphology, viability, and proliferative activity (21). For example, Rat1 cultures consist of viable cells only, whereas M1 aggregates are typically characterized by centrally located cell debris (Fig. 1). Also, the viable cell rim is ~100 µm thicker in MR1 spheroids than in Rat1-T1 spheroids (304 ± 24 vs. 204 ± 28 µm), and necrosis develops at a larger size (800–900 µm in MR1 spheroids vs. 500–600 µm in Rat1-T1 spheroids) (21).

The objective of the present study was to clarify whether ras transfection and tumorigenic conversion of fibroblasts directly leads to systematic changes in metabolic milieu and cell metabolism, as indicated in monolayer culture and tumor xenografts (13, 14, 20, 24). One particular aim was to analyze average cellular oxygen consumption rates as well as glucose, lactate, and ATP levels within the viable region of individual spheroids of the immortalized fibroblast cell lines Rat1 and M1 and of the highly tumorigenic ras-transformed fibroblast clones Rat1-T1 and MR1 relative to their growth/proliferation pattern and to their tumorigenic state.

MATERIALS AND METHODS

Cell Lines

Four differently transformed, diploid fibroblast cell lines derived from primary/secondary Fisher 344 rat embryo fibroblasts (REF) were used for the present investigation (24). Spontaneously immortalized (Rat1) and c-myc-transfected (M1) REF represent early, nontumorigenic stages in the transformation process and are characterized by poor cellular aggregation and aggregate growth in vitro (maximum diameters 150–300 µm) (21). Transfection of Rat1 cells with a point-mutated Ha-ras oncogene and c-myc/T24Ha-ras co-transfection of REF resulted in the development of the two highly tumorigenic and aggressively growing cell clones Rat1-T1 and MR1, respectively. Both ras transfectants show rapid spheroid formation with high proliferation rates (21) and maximum spheroid diameters of >1,200 µm (Fig. 1). Transfections were carried out via calcium phosphate precipitation with plasmids pH06T1 containing an Ha-ras oncogene isolated from a human bladder carcinoma and pSVc-myc1 detected first in a mouse plasmacytoma. Routine methods for cell preparation, oncogene transfection, and oncogene protein expression have been detailed previously (24).

Spheroid Culturing

Aggregates were cultured under identical physiological conditions using a spinner flask technique. DMEM (Sigma Chemical) supplemented with 25 mM glucose, 5% (vol/vol) FCS, 10,000 IU/l penicillin, and 10 mg/l streptomycin (Flow Laboratory/GIBCO) was used as culture medium. Cells were maintained in a humidified incubator equilibrated with 5%
(vol/vol) CO₂ in air at 37°C. Spheroids were initiated from trypsinized, exponentially growing monolayer cultures by inoculating (1–2) × 10³ T24Ha-ras-transfected Rat1-T1 or MR1 cells and (1–2) × 10⁴ Rat1 or M1 cells in 15 ml medium/100 mm nonadherent petri dish. After an initiation phase of 3 days, aggregates were transferred into a 1-liter spinner flask containing 300 ml medium according to a procedure detailed earlier (21). All spinner cultures were grown at 75–100 rpm thereafter, and medium was replaced daily. The number of spheroids per flask was reduced gradually with increasing spheroid size to maintain a relatively constant cell count of ≤ 2 × 10³ cells/ml medium in the spinner flask.

For each spheroid investigated, the total spheroid volume was calculated from two orthogonal diameters that were quantified using an inverted microscope equipped with a calibrated reticle. Spheroid volume growth was routinely recorded and analyzed for a representative spheroid population.

Thymidine Labeling Index and Cell Count

Thymidine labeling indexes (TLI) in spheroids were determined using conventional autoradiography (for details, see Refs. 17 and 21). Aggregates were pulse labeled at 37°C for 15 min in complete medium containing 4 µCi/ml [3H]thymidine (sp act 20 Ci/ml; New England Nuclear, Dreieich, Germany), fixed, histologically processed, and prepared for autoradiography using a standardized dipping technique and processing of the film (film emulsion K2; Ilford, Mobberley, UK). The TLI was determined on hematoxylin and eosin-stained central sections of the spheroids by microscopic observation as the proportion (%) of labeled nuclei to total nuclei count. Cells were considered labeled if the grain count per nucleus exceeded 10, with an average background of ≤1 grain per nucleus.

In addition to automated cell counting following spheroid dissociation, cell volumes of cells isolated from aliquots of spheroids with defined sizes have been analyzed using the Schaefer System Casy-1 (Schaefer System, Reutlingen, Germany) to allow for the calculation of cell volume-related oxygen consumption rates (21). Cell counts per spheroid were also calculated in histological sections from the individual values of the viable spheroid volume and the cell number per section surface according to Webel and Gomez (46).

ATP, Glucose, and Lactate Concentration and Glucose and Lactate Turnover

Local metabolite concentrations within spheroids were assessed via microelectrodes based on quantitative bioluminescence combined with single-photon imaging as described earlier (34, 35). ATP and glucose concentrations were determined in 5-µm thick cryostat sections through the center of rapidly frozen spheroids. Each frozen cryostat section was covered with a specific enzyme cocktail. Enzyme reactions linked the substrate of interest to the luminescence of different luciferases. For ATP detection, luciferase from firefly lantern was used, whereas glucose and lactate were identified via luciferase from marine bacteria (for details see Ref. 34). Bioluminescence intensities were registered using an appropriate microscope (Axiohot; Zeiss, Oberkochen, Germany) and an automated imaging photon counting system (ARGUS 100; Hamamatsu, Herrsching, Germany). Calibration was carried out as detailed previously by assigning photon counts to luminescence of defined tissue homogenate standards. Metabolite concentrations of these standards were quantified either by HPLC or by standard enzymatic assays (32). Substrate concentrations were obtained in micromoles per gram with respect to tissue net weight.

For the determination of glucose and lactate turnover rates, 10⁴ to 10⁵ spheroids with a defined size were transferred into 100-ml spinner flasks and were incubated in 50 ml of supplemented medium under culture conditions. After 10 min and every 60 min thereafter over a period of 6 h, two independent samples of 100 µl of medium were taken from the spinner flasks containing Rat1-T1 or MR1 cultures. For Rat1 and M1 aggregates, 100-µl medium aliquots were collected every 12 h over a period of 3 days due to poor glucose consumption and lactate release. Aliquots were immediately mixed with 1 ml and 0.5 ml ice-cold 0.6 M perchloric acid for glucose and lactate measurements, respectively. Samples were pelleted after 15–20 min, and metabolite concentrations were analyzed using routine test kits (test combination Glucoquant and Monotest lactate; Boehringer Mannheim), utilizing the metabolites for the production of NADH and NADPH to be measured photometrically at 365 nm. Mean turnover rates were calculated from the increase and/or decrease in metabolite concentration per time interval.

Oxygen Consumption

Oxygen consumption rates (Q˙O₂) and Krogh's diffusion constants K₀, within spheroids were calculated from oxygen tension measurements using Whalen-type oxygen-sensitive microelectrodes. The experimental design and protocol for stepwise recording PO₂ profiles on radial tracks through the spheroid center have been published previously (e.g., Ref. 29). Using model functions derived from simulations of oxygen diffusion in tumor spheroids, PO₂ profiles were evaluated by non-linear regression analysis that was based on a Quasi-Newton algorithm (10). From these calculations, K₀, as well as mean oxygen consumption rates per spheroid and per unit of viable spheroid volume (Q˙O₂/spheroid) were obtained. The average cellular oxygen uptake rate (Q˙O₂o) was then calculated for each spheroid from the individual Q˙O₂/spheroid values and the estimated cell counts per spheroid. Here the total cell count per spheroid was taken into account for all spheroid types and sizes, although the oxygenated rim was some 75 µm thinner than the viable cell rim within MR1 spheroids with a diameter ≥1,000 µm. Assuming that cellular respiration is zero within this "non"-oxygenated area, consumption rates per cell would be underestimated by 10–15% within the oxygenated layer of large MR1 spheroids.

Statistical Analysis

Group differences were evaluated using a two-tailed t-test for unpaired observations. Correlations between two parameters were obtained via linear regression analysis. The dependence of some of the assessed parameters (i.e., thymidine labeling index, O₂ consumption rate, and lactate concentration; see Table II) on cell biophase diameter dependence of TLI, Q˙O₂, and Lactate Levels (on spheroid diameter) was not well approximated by a straight line. Rather, it appeared that this dependence was biphasic, i.e., below and above a common limiting diameter two distinctly different linear regressions seemed to apply. The existence of a statistically significant biphasic behavior of these parameters was tested by employing the bilinear regression analysis developed in the APPENDIX (Statistical Analysis of Biphasic Dependencies).

1 Viable cell rim oxygen consumption = Q˙O₂ × (viable volume)/(total spheroid volume) = Q˙O₂ × [1 – (d – 29 µm²)²/d²] for a spheroid of diameter d and viable rim thickness of 29 µm (cf. Spheroid and Cell Characteristics).
RESULTS

Spheroid and Cell Characteristics

In addition to the characteristic differences between the four cell lines mentioned in the Introduction, cell count in small M1 aggregates is four to six times less than in Rat1, Rat1-T1, and MR1 spheroids of the same size (Fig. 2A), which is partly due to centrally located cell destruction. Within Rat1-T1 and MR1 cultures, cell count per spheroid increased with spheroid size being significantly higher in Rat1-T1 vs. MR1 spheroids of equal diameter (21). Concomitantly, the cell volume measured 734 ± 109 µm³ in Rat1-T1 spheroids and was consistently less (P < 0.001) than in MR1 spheroids (931 ± 113 µm³), as documented in Fig. 2B. In neither case, cell volume changed significantly (P > 0.05) as a function of spheroid diameter. Cell volume of Rat1 cells isolated from aggregates was comparable with that of Rat1-T1 cells, and M1 cells were similar to MR1 spheroid cells; hence cell volume in 3-D cultures was not altered by ras transfection.

Figure 2C presents the thicknesses of the viable cell rim in Rat1-T1 and MR1 spheroids as a function of spheroid size, showing a slight decrease within Rat1-T1 spheroids (R = 0.413; P < 0.01) but no change with diameter in MR1 3-D cultures. M1 aggregates with

![Figure 2](http://ajpcell.physiology.org/)

- **A**: Solid lines, best fits to the data for Rat1-T1 and Rat1.
- **B**: Solid lines, linear least squares best fits to the data.
- **C**: Rat1-T1 and MR1.
- **D**: Solid lines, polygons describing biphasic pattern of TLI as detailed in RESULTS (see also Table 1); dashed lines, linear least squares best fits to the entire data sets.

\[
y = -0.069x + 766.6 \quad (R = 0.234)
\]

\[
y = 0.071x + 839.0 \quad (R = 0.232)
\]

\[
y = -0.072x + 275.5 \quad (R = 0.413)
\]

\[
y = -0.005x + 307.6 \quad (R = 0.034)
\]
central cell death were characterized by a thin, no more than 29 ± 7 µm thick outer layer of morphologically intact cells.

From cell counts as well as cell and viable rim volumes, the ratio of intra- to extracellular space was estimated. Resulting average extracellular space was 68 ± 8% in the viable regions of MR1 spheroids (n = 24) and 50 ± 14% in Rat1-T1 spheroids (n = 23). Unfortunately, the scatter in these data was too large to allow for any predictions about systematic changes during spheroid growth. Interestingly, cell density within the viable rims of M1 spheroids turns out to be two to three times less than in Rat1, Rat1-T1, and MR1 spheroids of the same size.

[3H]thymidine labeling within the four aggregate types had shown earlier (21) that 1) the proliferative activity is much smaller in Rat1 and M1 aggregates compared with the highly tumorigenic Rat1-T1 and MR1 fibroblast spheroids at diameters of 150–300 µm, and 2) with growth, volume-averaged TLI in the tumorigenic spheroid types drops from 30–40% to values <20% (P < 0.05), with MR1 spheroids exhibiting higher TLIs (and faster spheroid volume growth) than Rat1-T1 spheroids. The diameter dependence of the individual volume-averaged TLI values (%) is further analyzed in Fig. 2D. It demonstrates that thymidine labeling of Rat1-T1 and MR1 tumor spheroids continuously decreases with increasing spheroid size up to a characteristic diameter of ~380 µm for Rat1-T1 and some 970 µm for MR1 spheroids. For larger aggregate sizes, TLI remains essentially constant.

ATP, Glucose, and Lactate Levels and Glucose and Lactate Turnover

ATP concentration within the viable region of 3-D MR1 cultures remained constant (P > 0.05) as a function of spheroid size, whereas a significant (P < 0.001) decline in the ATP level was observed for Rat1-T1 spheroids (Fig. 3A). In Rat1 and M1 aggregates, ATP concentrations were lower by a factor of 2–3 compared with the fully transformed counterparts (P < 0.0001), even though the proportion of intracellular space was two to three times lower solely in M1 aggregates. However, Rat1-T1 spheroids with diameters of 1,000–1,300 µm, which exhibited a larger fraction of quiescent cells, had ATP concentrations in the viable cell rim of
almost as low as determined for the small aggregates of the corresponding precursor Rat1.

In parallel with the ATP levels, glucose concentration was significantly decreasing in Rat1-T1 (P < 0.01) but not in MR1 spheroids as a function of spheroid size (Fig. 3B; glucose concentration in the medium was 25 mM). Yet, no biologically relevant lack in glucose could be observed in any of the fibroblast spheroid types investigated. The average glucose level ranged from 18 to 20 µmol/g.

In contrast to the relatively moderate changes in ATP and glucose levels, lactate accumulated in the viable cell rim of both Rat1-T1 and MR1 cultures during spheroid growth (P < 0.01), increasing from 2–4 µmol/g in small nonnecrotic spheroids to 7–9 µmol/g in the viable cell rim of spheroids with a diameter of ≥1,000 µm (Fig. 3C). Within the size range of ~200–300 µm, lactate concentrations in Rat1 and M1 aggregates were 6–7 µmol/g and tended to be higher than in the ras transfectants.

Other than the metabolite concentrations (that at least exhibited similar tendencies), glucose and lactate turnover rates showed very striking differences between Rat1-T1 and MR1 spheroids (Fig. 4). Although glucose uptake and lactate release per cell both moderately increased with diameter in Rat1-T1 spheroids (P < 0.025 and P < 0.0025, respectively), these turnover rates in MR1 spheroids were found to decrease some 50% up to a diameter of ~1,000 µm (P < 0.005 and P < 0.025, respectively) and to remain essentially constant in larger aggregates. Absolute turnover rates in MR1 compared with Rat1-T1 cells were about two to three times larger in small aggregates and very close to each other in large ones. Due to the poor aggregation characteristics and the small average spheroid volume, glucose uptake and lactate release in Rat1 and M1 aggregates were extremely hard to measure and were found to be in the lower range of their corresponding tumorigenic descendants.

Oxygen Diffusion and Consumption

Krogh’s diffusion constants within aggregates are documented in Fig. 5A as functions of spheroid size. There was no significant correlation between oxygen diffusion coefficients and spheroid size (for details see Ref. 20).

Oxygen consumption rates per viable spheroid volume (QŠO₂) slightly but insignificantly decreased as a function of spheroid size in MR1 spheroids, whereas QŠO₂ significantly dropped by some 50% (P < 0.01) in Rat1-T1 spheroids over a diameter range of 200 to ~800–900 µm (Fig. 5B). Rat1 and Rat1-T1 aggregates of comparable size did not significantly differ in their volume-related oxygen uptake rates. However, if aggregates with similar proliferation rates (Rat1 aggregates vs. Rat1-T1 spheroids with a diameter of ≥1,000 µm) were compared, a significant (P < 0.001) decrease in the oxygen consumption rate per viable volume was observed after ras transfection, which is not due to limited oxygen availability as shown earlier (21). QŠO₂ calculated in M1 aggregates was significantly lower than in their highly tumorigenic counterpart MR1 (P < 0.001). However, M1 aggregates are special, in that cell density in their viable rim is exceptionally low.

As mentioned above, M1 spheroids consist of an extraordinarily thin viable cell rim surrounding a central region that is characterized by morphological disintegration in routine paraffin histology (21) but that does not represent the classical type of necrosis in spheroids. Most surprisingly, the radial oxygen partial pressure profiles in M1 aggregates continually dropped...
from the surface of the aggregates up to their centers and appeared to be absolutely smooth across the boundary between viable rim and region of cell debris (Fig. 6). Similarly, ATP gradients extended beyond the viable cell rim as well (data not shown). Mathematical analysis of \( P_O^2 \) profiles consistently indicated that oxygen was consumed within the entire volume of M1 aggregates, even in the central region. We conclude that the latter is characterized by some unexpected respiratory activity, the rate of which was not distinguishable by our regression analysis from \( Q_{svO^2} \) in the viable rim. Ongoing energy metabolism as well as the unusual morphological appearance suggest that the material found in M1 spheroid centers is different from necrotic cells indeed. Rather, cell destruction in M1 aggregates may result from programmed cell death, which has been shown to be induced by \( myc \) transfection in epithelial (e.g., Refs. 2, 12, 37) as well as in fibroblast (e.g., Refs. 2, 12, 37) cells. The apoptotic process produces membrane-bound cellular fragments with intact, functional mitochondria, which may be metabolically active despite the fact that the cells are "dead" and which therefore may explain the presence of respiration and ATP in regions without intact cells.

Interestingly, MR1 spheroids show a phenomenon that, in a way, is complementary to the one just described for M1 spheroids. Next to the central necrosis in large MR1 aggregates there is an ~75-µm-thick layer of viable cells that is completely depleted of oxygen and exhibits zero oxygen consumption (21).
To eliminate the effects of differences in cell density on volume-related oxygen uptake, Fig. 5C presents cellular oxygen consumption rates (Q\textsubscript{CO2}) that are based on individual Q\textsubscript{O2} values per spheroid and the cell count per spheroid estimated from the formerly described relation between spheroid size and cell count (21). In M1 aggregates, oxygen consumption of the viable cell rim had to be used instead of Q\textsubscript{O2}, in the entire spheroid because oxygen consumption was found not only in the viable rim but also in the structurally disintegrated center.

In Rat1-T1 and MR1 spheroids, Q\textsubscript{CO2} decreased up to a diameter of ~830 and ~970 µm, respectively, by 35–45% (P < 0.001). Further spherical growth (>830 and 970 µm, respectively) was associated with enhanced cellular oxygen uptake (P < 0.001). Comparison of partly vs. fully transformed cell lines showed that Q\textsubscript{CO2} was not significantly different if spheroids within the same size range were taken into account. However, due to the reduction in Q\textsubscript{CO2} as a function of spheroid diameter in Rat1-T1 and MR1 spheroids (which was paralleled by a decline in the TLI), cellular respiration in Rat1 and M1 aggregates was significantly higher (P < 0.001) than that of the corresponding descendants at growth stages at which comparably low TLI values are reached (spheroid diameter ~830 µm for Rat1-T1 and ~970 µm for MR1 spheroids). This difference in Q\textsubscript{CO2} between immortalized ancestors and their ras-transfected descendants vanished more and more with further growth of the transfectants (occurring at constant TLI; see Fig. 2D), so the very large aggregates statistically differed in neither TLI nor (in the case of M1/MR1 only) Q\textsubscript{CO2} from their nontumorigenic ancestors.

Differences between the correlations of Q\textsubscript{svO2} and Q\textsubscript{CO2} with spheroid size are mainly due to variations in Q\textsubscript{O2} values per spheroid and the cell count on individual Q\textsubscript{O2} values per spheroid and the cell count at growth stages at which comparably low TLI values are reached (spheroid diameter ~830 µm for Rat1-T1 and ~970 µm for MR1 spheroids). Because cell volume of Rat1-T1 and MR1 cells in spheroids is not correlated with spheroid diameter (Fig. 2B), it must be the ratio of extracellular to intracellular space that changes as a function of spheroid size. Histological observations qualitatively confirm this result.

### Joint Biphasic Diameter Dependence of TLI, \(\dot{Q}_{O2}\), and Lactate Levels

F-test analysis of joint straight-line and polygon fits to TLI, lactate levels, Q\textsubscript{O2}, glucose consumption, and lactate release in Rat1-T1 and MR1 spheroids (see Appendix (Statistical Analysis of Biphasic Dependencies)) revealed that polygons with a common limiting diameter (d\textsubscript{L}) fitted the data much better than straight line fits (P < 0.0001). The resulting limiting diameters were d\textsubscript{L,Rat1-T1} = 830 µm for Rat1-T1 and d\textsubscript{L,MR1} = 970 µm for MR1. To determine whether there was a significant biphasic behavior in each one of these parameters, similar F-test analyses were performed individually with d\textsubscript{L} kept constant. It turned out that TLI, Q\textsubscript{svO2}, Q\textsubscript{CO2}, and lactate levels as well as glucose consumption and lactate production (the latter two in MR1 spheroids only) all were better described by polygons than by straight lines (P < 0.0001, P < 0.002, P < 0.0001, P < 0.033, P > 0.7, P > 0.4, respectively, for Rat1-T1; P < 0.0001, P < 0.04, P < 0.0001, P < 0.01, P < 0.006, P < 0.032, respectively, for MR1). Neither for ATP nor for glucose concentrations (nor for glucose and lactate turnover rates in Rat1-T1) could a significant improvement of the polygon approximation over the straight line approximation be found, even if the algorithm was allowed to determine a d\textsubscript{L} that was optimized only for the individual data set under study. Polygons describing TLI, Q\textsubscript{O2}, and lactate concentration in Rat1-T1 and MR1 spheroids as shown in Figs. 2–5 are given in Table 1. In MR1 spheroids, the increase of Q\textsubscript{CO2} with size in

### Table 1. Parameters describing biphasic pattern of TLI, \(\dot{Q}_{O2}\), lactate levels, glucose uptake, and lactate release within Rat1-T1 and MR1 spheroids as a function of spheroid diameter

<table>
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<tr>
<th></th>
<th>TLI</th>
<th>(\dot{Q}_{svO2})</th>
<th>(\dot{Q}_{CO2})</th>
<th>[Lactate]</th>
<th>Glucose Uptake</th>
<th>Lactate Release</th>
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<td>Rat1-T1</td>
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<td>-6.71e\textsuperscript{-7}</td>
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<td></td>
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Units correspond to that given in axis legends of Figs. 2D, 3C, 4A and 4B, and 5B and 5C. Polygons are \(y_i = ax + b\) [for \(x = 830\) µm (Rat1-T1) and \(x = 970\) µm (MR1), respectively] and \(y_i = cx + d\) [for \(x > 830\) µm (Rat1-T1) and \(x > 970\) µm (MR1), respectively]. TLI, thymidine-labeling index; Q\textsubscript{svO2}, oxygen consumption rate per unit of viable spheroid volume; Q\textsubscript{CO2}, cellular oxygen consumption rate; R, correlation coefficient of \(y_i/y_j\); R\textsubscript{0}, correlation coefficient of linear least squares best fits to the entire data sets; NS, not significant.
the upper diameter range actually is even more pronounced than indicated by Fig. 5C because QcO₂ in large aggregates has been underestimated by probably 10–15%.²

DISCUSSION

The present, well-established cell system allows for systematically studying the impacts of spontaneous immortalization vs. myc transfection as well as additional ras transfections on cell metabolism. As a third independent variable, the effects of 3-D growth in tumor spheroids are assessed. To facilitate understanding of the complex interrelations between the four cell lines studied, Fig. 7 summarizes the results (along with corresponding monolayer data from Ref. 24) as percent changes in TLI, cell volume-related oxygen uptake (QcVO₂), lactate release (QcVLac), and concentrations of ATP ([ATP]), glucose ([Glc]), and lactate ([Lac]) as function of days in monolayer culture or of spheroid diameter, respectively.

Metabolic Effects of Spheroid Growth Stage and Proliferative Activity: Biphasic Dependence of Metabolism on Diameter

It was demonstrated with extremely high statistical significance (P < 10⁻¹²) that there is a joint biphasic rather than a linear dependence of a number of metabolism-related parameters on spheroid diameter. This indicates that, at some specific aggregate size (denoted limiting diameter, dₘ), a certain “switch” in cellular metabolism is triggered that induces some sort of metabolic readjustment by changing the pattern of subsequent metabolic alterations. Most notably, with growth beyond dₘ, QcO₂ swaps from a significant decrease to an increase, whereas TLI, lactate concentration, and glucose uptake and lactate release (the latter two only in MR1 spheroids) become constant with ongoing spheroid growth (Fig. 7, k, l, o, and p).

The mechanisms that have been proposed to bring about the first phase of dropping TLI in ras-transformed fibroblasts are numerous and not well understood (19, 23, 26, 41, 43). The accompanying decrease in QcO₂ as well as lactate accumulation (which is similar to observations from former studies) may be accounted for by changing energy demand with falling cellular proliferative activity (combined with altered supply conditions) and are discussed below. For the second phase as well as for the nature of the metabolic switch, more complex explanations need to be developed thereafter.

ATP concentration and proliferative activity. ATP concentration (and, in correspondence, volume-related QsvO₂; Fig. 5B) decreased ~50% during spheroid growth in Rat1-T1 cultures, whereas it remained constant in MR1 spheroids (Fig. 7, l and p). Thus the course of [ATP] follows one of two patterns described earlier. 1) The ATP distribution is independent of tumor growth activity (33) and hence of spheroid size and position within the viable rim. This behavior of MR1 aggregates was also seen in EMT6 spheroids (Ref. 7 and Kunz-Schughart LA, Walenta S, and Mueller-Klieser W, unpublished observations). 2) On the contrary, data on Rat1-T1, WiDr (human colon carcinoma), and different human rhabdomyosarcoma spheroids show a decrease of ATP concentration in the viable cell rim with spheroid size (unpublished observations). This may result from a factual dependence of intracellular [ATP] on proliferative activity (36) or may be an artifact caused by falling cell packing density during growth. Alternatively, a fall in [ATP] may be indicative of beginning energy depletion enforcing adaptive metabolic actions for further growth (see Metabolism in large spheroids and metabolic trigger).

In Rat1 and M1 aggregates, [ATP] was lower than in the corresponding tumorigenic descendants (P < 0.0001; Fig. 7d and h vs. l and p). For M1 aggregates this may partly be explained by extremely poor cell viability in 3-D culture and very low cell packing density within the viable rim. TLI was low (~10%) in M1 and Rat1 aggregates, supporting the hypothesis that ATP levels might depend on proliferative activity. Similar changes of [ATP] with TLI have also been demonstrated in confluent vs. exponentially growing monolayer cultures of the four cell lines investigated (Doetsch and Mueller-Klieser, unpublished observations) (Fig. 7, b, f, j, and n).

Oxygen consumption and proliferative activity. In Rat1-T1 and MR1 spheroids smaller than dₘ, QsvO₂ decreased as a function of size (Fig. 7, k and o). This result corresponds with previous investigations, describing correlations of cellular respiration and proliferative activity during spheroid growth (1, 4, 8, 9, 20, 29, 44) as well as across viable rims of spheroids at given growth stages (6). In aggregates larger than dₘ, cellular respiration increases with spheroid diameter,² which came unexpectedly and is at variance with earlier studies (1, 8, 9, 29, 44). Although the fall in oxygen consumption for smaller spheroids is attributable to the reduced proliferative activity (Figs. 2D and 7, k and o), the same rationale would suggest QcO₂ to stagnate as TLI remains fairly constant at ~15% (Fig. 2D). Yet, this is not the case. Moreover, a strict dependence of QcO₂ on TLI is not supported by a regression analysis of QcO₂ vs. TLI (taken from Fig. 5C and calculated from the regressions given in Table 1, respectively). While there is a weak (R = 0.65) but statistically significant correlation in Rat1-T1 spheroids, QcO₂ and TLI are entirely unrelated in MR1 aggregates (R = −0.007, P > 0.95).

Glucose/lactate metabolism and proliferative activity. TLI and hence energy demand both become less during spheroid growth (Fig. 7, k and o). Therefore, not only QcO₂ but also glucose uptake rate should fall as well. With lengthening diffusion distances, diffusion limitation for lactate may occur (this is somewhat

²It should be noted that, in large MR1 spheroids, overall mean cellular oxygen consumption was observed to rise. This average has been taken over the whole 304-µm-thick and partly anoxic viable cell layer rather than the "respiring" cell rim that measured only 225 µm and consisted of 10–15% fewer cells (cf. Oxygen Consumption). Hence the rise in QcO₂ of well-oxygenated MR1 cells is even bigger.
Fig. 7. Synopsis of most important results for 4 cell lines studied (nontumorigenic ancestors in back, ras-transformed descendants in front). For each cell line, monolayer data (obtained from 24) are displayed as functions of time in culture (left panels), whereas data in spheroids have been calculated from best fits (cf. Figs. 2–5 and Table 1) and are given as functions of spheroid diameter (right panels). All data are presented as (averaged) percent changes relative to values of respective quantities calculated for 200-µm spheroids of the tumorigenic cell types (Rat1-T1 and MR1). Data sets include TLI, cell volume-related oxygen uptake (Q˙cvO2) and lactate release (Q˙cvLac), and concentrations of ATP ([ATP]), glucose ([Glc]), and lactate ([Lac]) (the latter two not for monolayers because they hold information about tumor cell metabolism only in spheroids). Monolayer ATP concentrations were adjusted to account for extracellular space that had entered spheroid data. For clarity, glucose uptake rates [which largely parallel lactate release rates (Fig. 4)] were omitted. Due to the enormous changes of cell volumes during monolayer growth (24), cell-volume-related turnover rates Q˙cvO2 and Q˙cvLac rather than Q˙cO2 and Q˙cLac are displayed.

Essential conclusions are as follows. 1) There is a biphasic dependence of TLI, Q˙cvO2, Q˙cvGlc, Q˙cvLac (the latter only in MR1; see k and o), and [Lac] (l and p), on spheroid diameter in ras-transformed, highly tumorigenic Rat1-T1 and MR1 aggregates, indicating some sudden switch in tumor cell energy metabolism. 2) During first phase, Q˙cvO2 falls in parallel with TLI, most likely reflecting a decrease in energy demand (k and o). 3) Lengthening of diffusion pathways leaves spheroid glucose concentration largely unchanged but causes lactate accumulation (l and p), which may, during further growth, exert some metabolic effect by impeding glycolysis and facilitating oxidative phosphorylation. 4) In a second phase, Q˙cvO2 sharply rises at stagnating proliferation rates, Q˙cvGlc, Q˙cvLac (in MR1; see k and o), and [Lac] (l and p), which may be due to induction of some energy-consuming process. 5) In aggregates and confluent monolayers of ancestors Rat1 and M1, Q˙cvO2 is much higher than expected from Rat1-T1 and MR1 cells at same TLI and under comparable culture conditions (a, c, e, g vs. i, k, m, o). 6) In contrast, very large Rat1-T1 and MR1 spheroids exhibit similar Q˙cvO2 and TLI as their nontumorigenic counterparts (c and g vs. k and o). 7) In confluent Rat1 and M1 monolayers, an increase in Q˙cvO2 at falling TLI (similar to 2nd phase of Rat1-T1 and MR1 spheroid cultures) is observed (a and e), which is not present (or much less pronounced) in ras-transformed descendants (i and m), suggesting that, after ras transfection, more intense stimuli are required to trigger the above metabolic switch. 8) There are also fundamental differences between the 2 tumorigenic cell lines that may be an effect of myc transfection: whereas MR1 energy production appears to be largely glycolytic to begin with (Q˙cvGlc and Q˙cvLac are high in monolayers and small aggregates and fall during spheroid growth with TLI, energy demand; and Q˙cvO2, [ATP], and [Glc] remain largely constant; see m, o, and p), anerobic glycolysis in Rat1-T1 seems to compensate for progressively corrupted oxidative ATP production during spheroid volume growth (low Q˙cvGlc and Q˙cvLac in monolayers and small spheroids that are enhanced when aggregates grow and Q˙cvO2 and [ATP] are falling; see i, k, and l).
Metabolism in large spheroids and metabolic trigger. In large Rat1-T1 (>630 µm) and MR1 aggregates (>790 µm), oxygen consumption increases with spheroid size (Figs. 7, k and o). This may be interpreted as a response to blockade of glycolysis by increased spheroidal lactate concentration (Figs. 3C and 7, I and p) that forces energy metabolism to favor oxidative pathways (in combination with rising energy demand for maintaining cellular integrity in an increasingly acidotic environment). Raising the proportion of aerobic vs. anaerobic energy production should, vice versa, attenuate further elevation of lactate concentration. This hypothesis is supported by Ref. 3, showing an increase in QO2 and even a net uptake of lactate with rising external lactate concentration. On the other hand, this reasoning is not completely satisfactory, since, below the limiting diameter, QO2 and lactate concentration are indirectly related, and, simultaneously with the rise in QO2, lactate concentration begins to stagnate rather than continue to increase, as postulated above (slope statistically indistinguishable from zero; Fig. 7, k, I, o, and p).

We conclude that there must be other causes for metabolic readjustment to occur. As there is no reason to believe that energy production might become uncoupled from ATP expenditure, possible energy-consuming processes that are initiated at the limiting diameter need to be considered. In normal fibroblast cells, e.g., during wound healing, time periods of augmented proliferation are followed by largely quiescent periods of differentiation in which collagen and other extracellular matrix (ECM) components are synthesized (5), necessitating extra energy consumption. In the malignant fibroblast cell lines, Rat1-T1 and, even more prominently, MR1, some rudimentary switch from a "state of proliferation" to a "state of differentiation/synthesis" may be preserved that is operated at dL, causing the rise in QO2. In addition, QO2 may be affected by an increased energy demand in preparation of apoptotic cell death (18), which may be induced by a more and more adverse environment (39).

Enhanced cellular oxygen uptake associated with not only stagnating but even falling proliferative activity has been found earlier in Rat1 and M1 monolayer cultures (24) (Fig. 7, a and e). As summarized in Table 2, QO2 decreased by >50% or >30%, respectively, during exponential growth, but rose to almost the original values in the confluent, contact-inhibited monolayer (TLI < 5%). Because cell volumes of all four cell types decreased as a function of time in monolayer culture, respiratory enhancement at confluence is even more pronounced for (volume-related) QcO2 (P < 0.0001; Fig. 7, a and e). In MR1 monolayers, TLI drops to ~15% only (and therefore the metabolic readjustment may be less conspicuous) and QcO2 remains largely unchanged, but QcO2 still rises significantly at confluence (P < 0.0001; Fig. 7m). In contrast (and in correspondence to the lesser rise in QcO2 in Rat1-T1 vs. MR1 spheroids), a QO2 increase in confluent Rat1-T1 monolayer cells was not seen; however, TLI was >25% even at confluence, which might not represent a sufficiently effective cell arrest (Fig. 7i).

Our observations suggest that 1) some of the mechanisms switching fibroblast cell metabolism between the states of proliferation and differentiation appear to be preserved, at least in rudimentary form, in immortalized as well as in ras-transfected, tumorigenic descendants; 2) on an adequate trigger (which may be related to metabolic micromilieu, intensity of cell-cell interactions, or the like) these mechanisms render proliferative activity to stagnate, QO2 to increase, etc.; 3) the switch is triggered more readily (i.e., already in the microenvironment of monolayers with only 2-dimensional interactions) in the immortalized than in the tumorigenic fibroblasts (probably due to suppression of cell differentiation and synthesis of ECM by ras transfection; see below). Consequently, metabolic readjustment is expected to be fully operative even in the smallest immortalized aggregates (which therefore consume oxygen at a rate much higher than predicted from the TLI), and hence the process of switching states can be observed in Rat1-T1 and MR1 spheroids (and in Rat1 and M1 monolayers) but not in Rat1 and M1 aggregates.

Metabolic Effects of Myc and Ras Transfections

Metabolism and tumorigenic conversion. In monolayers during early exponential growth and confluence, different for glucose). As a consequence, one should expect lactate to accumulate, which, in turn, may obstruct glucose turnover and energy production, may lower ATP levels, and may ultimately arrest proliferative activity (Ref. 3 and see below) and suppress cell viability. In the present study, lactate concentration ranged to 6–9 mM in the viable cell rim of Rat1-T1 and MR1 spheroids and to ~6 mM in Rat1 and M1 aggregates (Figs. 3C and 7, I and p). Lactate accumulation of comparable magnitude is known to impede cellular proliferation and energy metabolism [i.e., glycolysis (3, 11)] and to affect viable and proliferative rim thicknesses (3, 27). Despite similar lactate concentrations, only MR1 spheroids conform to the above pattern of attenuated glucose and lactate turnover with growth (Fig. 7o). In contrast, Rat1-T1 turnover rates increase (Fig. 7k), which may be interpreted as an adaptive mechanism as discussed below.

3 Our findings indicate that energy metabolism during growth is not affected by glucose depletion. Glucose concentration was held constantly high at 25 mM in the medium and was found in the center of the viable cell rim to drop during the observation period of spheroid growth from ~20 mM to values not less than 13 mM. Moreover, even in the spheroid center, glucose concentrations were >2.5 mM in Rat1-T1 and MR1 and >13 mM in Rat1 and M1 spheroids (data not shown). Physiological glucose levels, on the other hand, are 2.5–6 mM in the plasma and considerably less next to tissue cells located remote from capillaries.
cellular oxygen consumption rates ($\dot{Q}_{cO2}$) were lower in Rat1-T1 and MR1 cells than in the non-ras-transformed precursors (P < 0.001; Ref. 24; Table 2). The values of $Q_{cvO2}$ become equally smaller by ras transfection (Fig. 7, a and e vs. i and m). Hence there must be some extra energy sink in Rat1 and M1 cells that is unrelated to proliferation, suggesting that ras transfection per se corrupts differentiation and synthesis, which, in turn, may explain why the metabolic readjustment is fully effective even in Rat1 and M1 monolayers and small spheroids.

The present study indicates that $Q_{cO2}$ is also higher in Rat1 and M1 spheroids than in the corresponding ras transfecteds if aggregates with similar TLIs (and probably similar energy demands for proliferation) are compared (Fig. 7, c and g vs. k and o; near-limiting diameters). At unchanged TLI, this difference progressively disappears with further growth. This may readily be explained by the extra energy sink that is just being activated by metabolic readjustment in Rat1-T1 and MR1 aggregates while it was active in Rat1 and M1 monolayers and small spheroids.

Metabolism and myc transfection. Although by the current data major differences between Rat1 and M1 metabolisms cannot be identified, there are pronounced metabolic variations in energy metabolism. In Rat1-T1, worsening of oxygen supply conditions during spheroid growth (indicated by the drop in central PO2 (21)) leads to partial energy depletion ([ATP] decreases and hence TLI decreases and $Q_o$ decreases; Figs. 3A and 7, k and l) and shifts energy production toward the glycolytic pathway. Consequently, glucose and lactate turnover increase, rendering concentration gradients between medium and tissue larger (thus viable rim glucose concentrations decreases and lactate concentration increases; Fig. 7i), which represents a largely normal tissue response to hypoxia. In contrast, MR1 energy metabolism more heavily relies on anaerobic pathways to begin with. Therefore, it is not surprising that not only $Q_o$ but also anaerobic energy metabolism are found to decrease with falling TLI and energy demand (Fig. 7o). The consequences of a decrease in substrate turnover on concentration gradients between medium and tissue are opposite to those of spheroid growth; therefore, (other than in Rat1-T1) changes in glucose concentration with size turn out to be insignificant (Fig. 7p).

The striking differences between Rat1-T1 and MR1 glucose and lactate turnover rates (Figs. 4 and 7, k and o) may be interpreted as a consequence of some fundamental variations in energy metabolism. In Rat1-T1, worsened O2 supply conditions during spheroid growth (indicated by the drop in central PO2 (21)) leads to partial energy depletion ([ATP] decreases and hence TLI decreases and $Q_o$ decreases; Figs. 3A and 7, k and l) and shifts energy production toward the glycolytic pathway. Consequently, glucose and lactate turnover increase, rendering concentration gradients between medium and tissue larger (thus viable rim glucose concentrations decreases and lactate concentration increases; Fig. 7i), which represents a largely normal tissue response to hypoxia. In contrast, MR1 energy metabolism more heavily relies on anaerobic pathways to begin with. Therefore, it is not surprising that not only $Q_o$ but also anaerobic energy metabolism are found to decrease with falling TLI and energy demand (Fig. 7o). The consequences of a decrease in substrate turnover on concentration gradients between medium and tissue are opposite to those of spheroid growth; therefore, (other than in Rat1-T1) changes in glucose concentration with size turn out to be insignificant (Fig. 7p). The increase of lactate concentration in spheroids smaller than $d_o$, on the other hand, is significant (P <

### Table 2. Average cell numbers per surface cell volumes, TLI, and $\dot{Q}_{cO2}$ of rat embryoid fibroblasts transformed to different extents in exponential and confluent monolayer culture

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Confluent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell count/cm² ($\times 10^4$)</td>
<td>Cell volume, µm³</td>
<td></td>
</tr>
<tr>
<td>Rat1</td>
<td>0.24 ± 0.02</td>
<td>1.57 ± 0.04</td>
<td>4.43 ± 0.15</td>
</tr>
<tr>
<td>Rat1-T1</td>
<td>0.26 ± 0.01</td>
<td>3.36 ± 0.16</td>
<td>37.2 ± 0.41</td>
</tr>
<tr>
<td>M1</td>
<td>0.23 ± 0.01</td>
<td>4.51 ± 0.19</td>
<td>12.5 ± 0.79</td>
</tr>
<tr>
<td>MR1</td>
<td>0.37 ± 0.05</td>
<td>7.56 ± 0.60</td>
<td>59.2 ± 1.51</td>
</tr>
</tbody>
</table>

Values are means ± SD. For comparison, TLI and $\dot{Q}_{cO2}$ in Rat1-T1 and MR1 spheroids of diameters ≤400 µm, limiting diameter (830 µm in Rat1-T1 and 970 µm in MR1, respectively), and >1,200 µm have been calculated.
Changes in Q˙svO2 with radial position are not quantified. In the present study, average respiration rates in the Q˙svO2 distributions is not satisfactory, given the limited method's sensitivity for determining spatially resolved precision of currently available input data. Neverthless, substantial radial gradients in QsvO2 can be detected because, if present, they ensue pronounced systematic deviations of the best approximations from the measured PO2 profiles. No such deviations were observed, hence QsvO2, most likely was rather homogeneous, although there is experimental evidence suggesting a drop in mitochondrial function and possibly also QO2 from outer to inner regions in Rat1-T1 and MR1 spheroids (22).

The above limitation has consequences for the interpretation of QsvO2 in M1 spheroids. As described in RESULTS, PO2 gradients as well as [ATP] drops extended beyond the viable cell rim and far into the central region, both being indicative of a persisting metabolic activity despite structural disintegration. Central QsvO2 was not distinguishable from QsvO2 in the viable rim by our analysis, and the reported value represents a spatial average over the entire M1 spheroid volume. In this particular case, it may be possible that even sizable differences between QsvO2 in the viable cell rim and central region were not detected, as dimensions of and number of data points within M1 viable rims are extremely small, rendering the method rather insensitive. Thus QO2 in viable M1 cells may be higher, which may, in part, explain the extraordinarily low QsvO2 values found in M1 aggregates (Fig. 5B).

Cell volume and QcO2 in spheroid vs. monolayer culture. Spheroid cell volume has been found to be independent of time in culture, proliferation rate, and malignant transformation (not, however, of myc transfection). In monolayers of each cell line, cell volume is directly related to TL1 and decreases with time in culture (which may be caused by changing intensity of contact inhibition with number of cells per dish). If cell volume-TL1 relations of different cell lines are compared, there is an indirect relation, i.e., larger cells are associated with lower TL1 and vice versa. These observations suggest that, in early monolayers (with plenty of space available), there is little control of cell volume by contact inhibition but rather by the demands of proliferation, whereas, at confluence, the available space seems to be a major determinant.

QcO2 in confluent M1 and Rat1 monolayers is two to three times that observed in spheroids (Table 2). This is partly a consequence of 2-D culture, as cell volumes are 50–100% larger. Given some systematic deviation due to different techniques for determining QO2 (model evaluation of PO2 profiles vs. tracking medium oxygen concentration drop by photometry), QO2 per cell volume may be better compatible under both culture conditions.

Metabolic readjustment in spheroid vs. monolayer culture. As already mentioned, the fall and, later on, rise of QcO2 in growing M1 and Rat1 monolayer cultures parallels the courses of QcO2 in MR1 and Rat1-T1 spheroids, suggesting that in both cases similar metabolic readjustment occurs. It seems that the more malignant the cell line, the more intense cell-cell interactions and/or hostile metabolic milieu is necessary to trigger the metabolic switch. Although in M1 and Rat1 cells the 2-D contact inhibition in monolayers suffices to induce complete readjustment, this occurs for the ras-transformed descendants only in 3-D culture and under rather adverse environmental conditions. In consequence, some cell line specific characteristics of malignant cells obviously become expressed only under very particular conditions that are present, e.g., during volume growth in large spheroids and probably also in vivo (3-D contact inhibition and/or detrimental metabolic milieu), but that are never attained in monolayers.

Relevance for tumors in vivo. In the preceding subsections on cell volume and metabolism in spheroids vs. monolayers, it was shown that, under 3-D growth conditions (present in vivo and in multicellular spher-
strategies for tumor chemotherapy. Malignant tumors and may serve as a basis for new elucidation of the exact nature, the triggering conditions, and clinical aspects. Yet, it cannot necessarily be expected that findings from the present in vitro model are directly applicable to the metabolism of ras-transfected human tumors because, among other reasons, the latter are generally of epithelial origin, whereas the former are derived from fibroblast cells.

In conclusion, our investigations have shown that myc but not ras transfection per se seems to have a major impact on fibroblast metabolism. In the late growth stages, some striking metabolic readjustment was identified that comprised reversal of the observed fall in \( Q_{O2} \) with growth accompanied by stagnant proliferative activity. While in the immortalized cell types this metabolic switch was triggered in monolayer culture already, in the ras-transfected cells the spheroidal 3-D environment was required to induce similar metabolic changes. Further studies will be necessary to elucidate the exact nature, the triggering conditions, etc., of this metabolic switch. This knowledge may be helpful to better understand the complex metabolism of malignnt tumors and may serve as a basis for new strategies for tumor chemotherapy.

**APPENDIX**

Oxygen Diffusion and Consumption

\( P_{O2} \) distributions in and around multicellular tumor spheroids suspended in stirred media may be recorded by using oxygen-sensitive microelectrodes and are characterized by constant \( P_{O2} \) in the stirred medium and by a continuous \( P_{O2} \) decline in the unstirred layer surrounding the spheroid (oxygen-depleted zone) as well as in the spheroid itself. This type of \( P_{O2} \) distribution may be described mathematically by diffusion theory. \( P_{O2} \) profiles within spheroids are governed by spheroid geometry and by the ratio of volume-related oxygen consumption rate (\( Q_{svO2} \)) over Krogh’s diffusion constant (\( K_{O2} \)). Hence, for known spheroid geometry, the latter ratio may be computed by fitting mathematical representations of \( P_{O2} \) profiles in spheroids to experimental ones. To determine \( Q_{svO2} \) and \( K_{O2} \), separately, additional information is needed that, on principle, may be obtained from the magnitude of the break in the \( P_{O2} \) profile at the spheroid surface. Practically, one may use a mathematical algorithm for nonlinear minimization to approximate the experimental \( P_{O2} \) profiles (and their gradients) inside and outside the spheroid by theoretical \( P_{O2} \) distributions to find \( Q_{svO2} \) and \( K_{O2} \). Because the errors in the gradients derived from measured \( P_{O2} \) profiles are generally larger than the measuring errors in the \( P_{O2} \) itself and since, in addition, distortions of the electrode signal near the interface between medium and spheroid are frequent, the accuracy of individual estimates of \( Q_{svO2} \) and \( K_{O2} \) is much lower than that for determining only the ratio \( Q_{svO2}/K_{O2} \). Accordingly, an enormous scatter is found in \( Q_{svO2} \) and \( K_{O2} \) that is not present in \( Q_{svO2}/K_{O2} \). From large compilations of oxygen diffusion coefficients (e.g., Ref. 42), there is good evidence that the diffusion properties of biological tissues are not highly variable and that \( K_{O2} \) mainly depends on the water content of the tissues. Because the latter should be relatively constant in spheroids of the same cell line and similar size, it was decided that most of the scatter in \( Q_{svO2} \) and \( K_{O2} \) computed individually (that was indeed much larger than the scatter in \( Q_{svO2}/K_{O2} \)) was caused by technical problems and was not indicative of any biological variation in \( K_{O2} \). Therefore, after a first evaluation of \( Q_{svO2} \) and \( K_{O2} \) in individual spheroids, a relation between average \( K_{O2} \) and spheroid diameter was determined from a linear least-squares fit to the individual \( K_{O2} \) values over spheroid diameter. This relation was employed to determine a fixed, scatter-free \( K_{O2} \) for each spheroid diameter that was then used to reevaluate \( Q_{svO2} \) in every single aggregate from the ratio \( Q_{svO2}/K_{O2} \). In this way, the scatter in the resulting \( Q_{svO2} \) was reduced to that in the ratio \( Q_{svO2}/K_{O2} \) (at the cost of a possibly somewhat less precise average value).

**Statistical Analysis of Biphasic Dependencies**

As stated in Statistical Analysis, the dependence of some of the assessed parameters appeared to be biphasic, i.e., below and above a common limiting diameter two distinctly different linear regression lines seemed to be necessary for an optimal fit. On the other hand, whenever two independent linear least-squares fits were used for the ranges of small and large spheroid diameters, a discontinuity at the limiting diameter was almost certain to be present, which one would not expect to exist in the functions relating these parameters to spheroid diameter, neither from the biological background nor from obvious evidence. Therefore, in selecting a type of expression function that was better suited to fit the data, a polygon was chosen that consisted of two straight-line segments that intersected at the limiting diameter. Such a polygon is uniquely defined by the limiting diameter and three additional parameters specifying slopes and intercepts of the polygon segments. For the polygonal expression functions, the sums of squares of weighted deviations from the measured data were minimized by determining one optimal limiting diameter as well as three more parameters for each of the polygons approximating TLI, \( Q_{svO2} \), and lactate levels.

Evidently, different diameter dependencies of a number of parameters in small and large spheroids may be reflections of differences between biological properties in each of the two size ranges. However, for any physiological interpretation of this phenomenon to be feasible, it first needs to be shown that the described biphasic relations actually do exist and are more than merely subjective impressions. This is performed according to Motulsky and Ransnas (28) by statistically comparing the goodness of the approximation of the data by straight lines or by polygons using an F-test analysis. As approximations based on a larger number of variable parameters obviously tend to yield better results than fits based on fewer ones (no matter if they really furnish a better description of the actual biological dependence or not), the different numbers of fit parameters (6 for 3 straight-line fits in the first case and 9 for the 3 polygon fits plus 1 limiting diameter in the 2nd) and, accordingly, the degrees of freedom need to be considered to make a valid comparison.

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