Low Mitochondrial Proton Leak Due to High Membrane Cholesterol Content and Cytosolic Creatine Kinase as Two Features of the Deviant Bioenergetics of Ehrlich and AS30-D Tumor Cells

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ABSTRACT

Isolated mitochondria from highly glycolytic Ehrlich and AS30-D tumor cells have a 12.4- and a 2.3-fold higher cholesterol level, respectively, than that of rat liver mitochondria. The passive proton permeability of Ehrlich and AS30-D tumor inner membrane mitochondria is, respectively, 4- and 1.4-fold lower than that of rat liver mitochondrial membrane. This feature is accompanied by a lower proton leak current in tumor mitochondria. A 3.5-fold cholesterol enrichment of rat liver mitochondria decreases their passive proton permeability by a factor of 2, thus establishing a direct relationship between the cholesterol contents of mitochondrial membranes and the passive proton permeability. Creatine kinase activity is present in the cytosol of these cells and is mostly represented by the BB isoform. Since AS30-D tumor cells' treatment with the creatine analogue β-guanidinopropionic acid decreases their life span and viability, creatine kinase is an indispensable enzyme entering a main energy distribution pathway starting from mitochondrial ATP, through glycolysis and creatine phosphorylation, to satisfy the large energy demands of tumor cell division.

INTRODUCTION

Cancer cells have a deviant energetic metabolism. A high glycolysis is related to a high degree of dedifferentiation correlated with the appearance of a variety of isozymes. In intact glycolytic tumor cells, only 10% of the glycolytic pyruvate enters a truncated Krebs cycle (1): citrate is preferentially extruded from mitochondria to the cytosol (2), where its two-carbon units (acetyl-coenzyme A) feed an already deregulated (increased) cholesterol synthesis (3-5). A flux of C4 compounds to pyruvate, acetyl-coenzyme A, and citrate is indispensable for the complete oxidation of glutamine, considered to be the major respiratory substrate of these glycolytic tumor cells (6), to CO₂ and water through the abnormal Krebs cycle. This flux is favored by the presence of the tumor progression-linked intramitochondrial NAD(+)/NADH malic enzyme that traps the extramitochondrial malate to decarboxylate it into pyruvate + CO₂ (7).

We have previously shown that glycolytic tumor cells were able to produce an unusual product for mammalian cells, acetoin, from an elevated nonoxidative decarboxylation of pyruvate (8). Acetoin added to tumor mitochondria increases extramitochondrial citrate levels (9) in a way that could account for the global increase of cholesterol contents. Cholesterol accumulation in membranes changes their biophysical properties. We have examined to what extent such a cholesterol increase in mitochondrial membranes from normal and tumor cells may influence proton permeability and thus the ability to make ATP. Recently, Arora and Pedersen (10) have shown that a hexokinase isozyme, bound to the outer mitochondrial membrane, contributes to the continuous feeding of tumor glycolysis through glucose phosphorylation. Most of the energy produced in glycolytic cancer cells, which comes from glycolytic ATP, is mainly devoted to cell divisions. In a cell that is known to dissipate energy, some mechanism must exist that makes the link between mitochondrial ATP (mainly recycled through bound hexokinase to feed glycolysis) and cytoplasmic ATP, this latter being efficiently dispatched to demanding mechanisms. Some CK³ isozymes have been found sometimes in cancer cells, which are not normally present in parental cells (11). We have investigated the possibility that the presence of CK isozymes in glycolytic tumor cells could be a necessity for the energetic distribution in the cells rather than a genetic deregulation due to the cancer process.

MATERIALS AND METHODS

Cell Lines and Animals. Ehrlich ascites tumor cells (spontaneous murine mammary carcinoma) and AS30-D ascites tumor cells (chemically induced rat hepatoma) are cultured in Dulbecco's modified Eagle medium enriched with 1 mm glucose and 10% heat-inactivated fetal calf serum according to the method of Baggetto and Lehninger (8). After 5 passages, 5 × 10³ Ehrlich cells and 200 × 10³ AS30-D cells are injected i.p. into young male Swiss mice and young female Sprague-Dawley rats, respectively (IFCA-Credo, Les Oncins). A week later, the ascitic fluid is collected and returned to culture.

When required, AS30-D cells have been cultured in the presence of either 10 mm sterile β-GPA (Sigma) or 20 mm creatine (Sigma).

Cellular Fractions and Enzymatic Dosages. Cell fractionation from 500 × 10⁶ living cells or from 3 g of adult rat liver is performed according to the following method. A general scheme summarizing the different steps is presented in Fig. 1. Cells are pelleted at 700 × g for 5 min at room temperature and suspended in a total volume of 50 ml with the H-medium containing 210 mm mannitol, 70 mm sucrose, 0.5% bovine serum albumin, 5 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mm EGTA (pH 7.2). After the addition of 5.5 mg digitonin in H-medium and homogenization at 0°C, when cell viability (checked with trypan blue) is less than 5%, the volume is doubled with H-medium. Cells are centrifuged at 3000 × g for 3 min at 4°C. The supernatant S2 contains the cytosol. The pellet P2 is suspended in 20 ml H-medium, homogenized with a tight Teflon pestle and glass homogenizer (Thomas type C) after 15 up-and-down strokes, and centrifuged at 625 × g for 5 min at 4°C. The following applies for both rat liver and ascitic cell preparation. The pellet P3 is washed in 20 ml H-medium after homogenization in the type C homogenizer. After centrifugation at 625 × g for 5 min (4°C), the pellet P4 containing nuclei and heavy particles is kept, while supernatants S3 and S4 are pooled and centrifuged at 9800 × g for 10 min at 4°C. The following applies for both rat liver and ascitic cell preparation. The pellet P5 is washed in 20 ml H-medium after homogenization in the type C homogenizer. After centrifugation at 625 × g for 5 min (4°C), the pellet P4 containing nuclei and heavy particles is kept, while supernatants S3 and S4 are pooled and centrifuged at 9800 × g for 10 min at 4°C. The following applies for both rat liver and ascitic cell preparation. The pellet P5 is washed twice in 1 ml H-medium and centrifuged at 9800 × g for 10 min at 4°C. Supernatants S6 are pooled, the pellet P6 containing clean mitochondria.

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3 The abbreviations used are: CK, creatine kinase; RLM, ATM, ETM, mitochondria from, respectively, rat liver, AS30-D, and Ehrlich tumor cells; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; β-GPA, β-guanidinopropionic acid; PCr, phosphocreatine.
mitochondria is suspended in 0.5 ml of H-medium. Postmitochondrial fractions are obtained by the centrifugation of P6 at 13,000 × g for 5 min at 4°C. The pellet P7 is suspended in 2 volumes of distilled water for 1 min at 0°C and centrifuged at 13,000 × g for 5 min at 4°C. The supernatant S8 contains outer mitochondrial membranes and intermembrane spaces is kept, and the pellet P8 containing mitoplasts, is suspended in the preceding volume of 100 mM Na2HPO4 (pH 7.3), for 15 min at 0°C. After centrifugation at 13,000 × g for 5 min at 4°C, the supernatant S9 contains the mitochondrial intermembrane space proteins not solubilized by water treatment, whereas the pellet P9 contains inner mitochondrial membranes plus matrix.

Cell fractions are checked for purity by measurement of the following marker enzymes: lactate dehydrogenase (cytosol) with a UV method (12) and citrate synthase (mitochondria) (13).

Creatine Kinase Activity. CK enzymatic activity is measured by the following "pH-stat" method (14) using an Autotitristop (Taucussel) apparatus and based on the protons formed when ATP is hydrolyzed during creatine phosphorylation catalyzed by CK. These protons are readily neutralized by the automatic injection of 10^{-2} N NaOH in order to keep a constant pH of 8.8 (optimum for the enzyme). The reaction medium contains 4 mM ATP, 5 mM magnesium acetate, 40 mM creatine, 0.05 mM EDTA, 0.05 mM dithiothreitol, and 50 mM potassium acetate. Mitochondrial ATPase activity is blocked by the addition of 2 μg oligomycin/assay. The oligomycin-insensitive ATPase activity is measured and subtracted from the total activity measured.

Creatine kinase isozyme activity is visualized by cellulose acetate electrophoresis described in (15).

Mitochondrial Incubations. Mitochondria have been prepared as mentioned above, according to the high-yield method described by Mo-rath and Fiskum (16), either from the ascitic cells or from rat liver (adult Sprague-Dawley), and kept in the H-medium at the highest concentration possible.

Mitochondrial incubations for oxygen consumption during substrate oxidation are performed in a 1.5-ml thermostatized Clark vessel with a platinum oxygen electrode. Measurements are done at 30°C in a 1-ml air-saturated respiratory medium containing 130 mM KCl, 10 mM morpholinooethanesulfonic acid, 2 mM MgCl2, and 2 mM KH2PO4/K2HPO4, under constant stirring. Unless otherwise indicated, 1 mg mitochondrial proteins is added, followed by 1 mM succinate as the respiratory substrate. Dissolved oxygen amounts to 444 nmol/ml of respiratory medium at 30°C.

Proteins. Proteins were measured by the biuret method (17) and by the method of Peterson (18).

Cholesterol Enrichment of Mitochondria. Cholesterol enrichment has been performed according to a slightly modified method of Coleman et al. (19). Sephadex-G10 beads (Pharmacia) (1 g) are washed in benzene, added or not to 36.6 mg cholesterol (Sigma), and dried under vacuum in a rotative evaporator. RLM (1 ml) prepared as described above and containing 40 mg proteins are incubated either with 125 mg benzene-washed Sephadex beads or with 125 mg cholesterol-coated Sephadex beads for 1 min at 0°C under gentle agitation. The mixture is deposited on top of 10 ml of a 60% sucrose cushion and centrifuged at 1600 × g for 10 min at 4°C. Mitochondria on top of the cushion are collected, suspended in H-medium, and centrifuged at 9700 × g for 12 min (4°C). The mitochondrial pellet is suspended in 1 ml of H-medium.

Cholesterol Measurement. Free and total cholesterol have been measured in mitochondrial fractions from Ehrlich or AS30-D tumor mitochondria and from intact or cholesterol-enriched rat liver mitochondria according to a fluorimetric method (20) adapted to our cell lines as described by Baggetto and Testa-Parussini (9).

Passive Proton Permeability of Mitochondrial Membranes. To determine the passive proton permeability of mitochondrial membranes from normal and cancer cells we have adapted the technique of Groen et al. (21) as follows. Mitochondrial proteins (1 mg) (ATM, ETM, intact or cholesterol-enriched RLM) are incubated in aerobiosis in 1 ml of the respiratory medium described above. Ten mM succinate is added as the substrate together with 3.4 μg rotenone/ml (to block site I of the respiratory chain), 1 μg oligomycin/ml (to block oxidative phosphorylations), and 4 × 10^{-5} M N-ethylmaleimide (to block the phosphate translocator). Substrate oxidation is progressively stimulated by the addition of increasing amounts of FCCP, giving the respiration rate V. This rate is compared to the rate of respiration stimulated to a maximal extent by the addition of 0.1 μM FCCP. Under these conditions, the relative rate of oxygen consumption (100 × V/V_{max}) is a linear function of the uncoupler amount, up to the FCCP amount for which V/V_{max} equals 1. Extrapolation of this relationship to V/V_{max} = 0 gives an amount that is directly related, in absolute values, to the passive proton permeability of the mitochondrial membranes. The slope of the line (k[FCCP]), which represents the mitochondrial sensitivity to FCCP, is related to the partition coefficient of FCCP on the membranes, to the cholesterol contents of the membranes, and to the proton conductance of the membrane.

The current of protons flowing back into mitochondria (I_{H+}) to short-circuit the proton current flowing around the classical chemiosmotic proton circuit [I_{H+}] may be readily evaluated from the rate of respiration induced by the addition of FCCP (dO/dt), the H+/O stoichiometry [6 for succinate as substrate (22)], and the factor k[FCCP]:

\[ J_{H+} = \frac{1}{k[FCCP]} \times \frac{dO}{dt} = H^+ \times O \] (nmol H+/min · mg proteins)

RESULTS

Cholesterol Content of Mitochondrial Membranes from Highly Glycolytic Tumor Cells. Table 1 shows that ETM have 12.4- and 13.9-fold higher levels of total and free cholesterol, respectively, than RLM. This is mainly due to an increase of nonesterified cholesterol distributed to the inner and, to a main
Experiments for AS30-D tumors, ±SE.

Fluorimetric method as mentioned in “Materials and Methods.” Results correspond to means of 5 experiments for Ehrlich tumors, 4 experiments for rat liver, and 3 experiments for AS30-D tumors, ±SE.

The extent, to the outer mitochondrial membranes. The latter contain 5.1-fold higher levels than RLM. ATM show a 2.3-fold increase in their total cholesterol content when compared to RLM, here, too, due mainly to an increase in free cholesterol content in both inner and, to a larger extent, outer mitochondrial membranes, where it is 3.5 times more elevated.

The 12.4- and 2.3-fold higher total cholesterol levels in mitochondrial membranes from highly glycolytic tumor cells as compared to RLM change the chemical structure of these membranes. The contorted shape of tumor mitochondria visualized by electron microscopy (not shown) could be related to the observed increase in mitochondrial membrane cholesterol content, which in turn affects their physicochemical properties.

Several bioenergetic characteristics could be affected by such membrane changes, among which are the proton leak through the inner mitochondrial membrane and mitochondrial phosphorylations.

Passive Proton Permeability of Mitochondria from Tumor Cells versus RLM. Proton leak through the inner mitochondrial membrane is one of the rate-limiting steps in the oxidative phosphorylation processes (23).

Fig. 2 shows the relative respiration rates ($100 \times V/V_{max}$) as a function of increasing amounts of the uncoupler FCCP added to stimulate the succinate oxidation rate by RLM, ETM, and ATM. Under our experimental conditions, the relative rate of oxygen consumption ($100 \times V/V_{max}$) is a linear function of the uncoupler amount, up to a ratio $V/V_{max} = 1$, for each type of mitochondria tested. The passive proton permeability is estimated by extrapolation of the linear relationship to a zero value of the ratio $V/V_{max}$. The passive proton leak through inner mitochondrial membranes is equivalent to 49.6, 35.1, and 12.5 pmol FCCP/ml and per mg mitochondrial proteins for, respectively, RLM, ATM, and ETM. Thus the passive proton leak of inner mitochondrial membranes of ETM and ATM is, respectively, 4-fold and 1.4-fold lower than in RLM.

The slopes of the lines obtained, $k(FCCP)$, represents the sensitivity of mitochondrial membranes to FCCP and is related to its partition coefficient. Their values are 0.33, 0.95, and 1.86, respectively, for RLM, ATM, and ETM.

We can calculate the flow of protons that leak back into the mitochondrion ($J_{H^+}$) and short-circuit the chemiosmotic proton circuit $J_{H^+}$ with the formula given in “Materials and Methods.” Assuming an $H^+$/O stoichiometry of 6, passive $H^+$ leak rates ($J_{H^+}$) would be 1145, 334, and 200 nmol $H^+$/min·mg proteins, respectively, in RLM, ATM, and ETM when the membrane electrochemical potential ($\Delta \mu_{H^+}$) is the highest (i.e., under maximal FCCP uncoupling). Therefore the relative reduction in passive $H^+$ leaks in the ATM and ETM amount to about 29.2% and about 17.5% of normal RLM.

One question arises as to whether the reduced passive proton permeability, and thus the reduced proton leakage flow in glycolytic tumor cell mitochondria, is related to the higher membrane cholesterol content.

Cholesterol Enrichment of Rat Liver Mitochondrial Membranes. Fig. 3 shows the effect of cholesterol enrichment of rat liver mitochondrial membranes on their passive proton permeability.

The values of $100 \times V/V_{max}$ plotted against the FCCP amounts used to stimulate succinate oxidation by RLM give, after extrapolation of the line $RLM$ to the abscissa axis, an equivalent of 49.6 pmol FCCP for passive proton permeability. The same batch of mitochondria 3.5-fold-enriched with cholesterol ($RLM$ Chol) shows a passive proton permeability equivalent to 25 pmol FCCP, or about 50% that of normal RLM. This value has been calculated after the effect of noncoated Sephadex beads on mitochondrial membranes (line $RLM^{+}$) had been subtracted from the effect of cholesterol-coated beads (line $RLM + Chol$).

It is clear that when rat liver mitochondrial membranes have a 3.5-fold cholesterol enrichment (from 0.43 nmol/mg proteins up to 1.5 nmol/mg proteins measured by fluorimetry), their passive proton permeability is 2-fold lower. From the formula given under “Materials and Methods,” when normal RLM are loaded with 3.5-fold more cholesterol, the passive proton leak ($J_{H^+}$) is reduced from 1145 to 573 nmol $H^+$/min·mg proteins, or 50%. Thus cholesterol enrichment of mitochondrial membranes is responsible for a lower passive proton permeability and thus a lower proton leak.

Efficiency of Mitochondrial Phosphorylations. A second bioenergetic characteristic of tumor cells that may be affected

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**Table 1 Total and free cholesterol content of mitochondrial fractions from rat liver and tumor cells**

Mitochondria and mitochondrial fractions are prepared as indicated in “Materials and Methods.” Free (nonesterified) and total cholesterol are measured by the fluorimetric method as mentioned in “Materials and Methods.” Results correspond to means of 5 experiments for Ehrlich tumors, 4 experiments for rat liver, and 3 experiments for AS30-D tumors, ±SE.

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>Inner membrane</th>
<th>Outer membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Free</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>(nmol/mg proteins)</td>
<td></td>
<td>(nmol/mg proteins)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>0.31 ± 0.03</td>
<td>0.09 ± 0.01</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>Ehrlich tumor</td>
<td>3.83 ± 0.12</td>
<td>1.25 ± 0.09</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>AS30-D tumor</td>
<td>0.70 ± 0.06</td>
<td>0.23 ± 0.02</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Passive proton permeability of mitochondrial membranes from tumor cells and rat liver. Mitochondrial proteins (1 mg) from rat liver (O), Ehrlich tumor (△) and AS30-D tumor (■) are incubated in 1 ml of the respiratory medium described in “Materials and Methods,” in the presence of 10 μm succinate as the substrate, 1 μg oligomycin, 3.4 μg rotenone, and 4 × 10⁻⁵ M N-ethylmaleimide. Respiration rate ($V$) is stimulated by the addition of increasing amounts of the uncoupler FCCP added to stimulate the succinate oxidation rate by RLM, ETM, and ATM. Under our experimental conditions, the relative rate of oxygen consumption ($100 \times V/V_{max}$) is a linear function of the uncoupler amount, up to a ratio $V/V_{max} = 1$, for each type of mitochondria tested. The passive proton permeability is estimated by extrapolation of the linear relationship to a zero value of the ratio $V/V_{max}$. The passive proton leak through inner mitochondrial membranes is equivalent to 49.6, 35.1, and 12.5 pmol FCCP/ml and per mg mitochondrial proteins for, respectively, RLM, ATM, and ETM. Thus the passive proton leak of inner mitochondrial membranes of ETM and ATM is, respectively, 4-fold and 1.4-fold lower than in RLM.

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Efficiency of Mitochondrial Phosphorylations. A second bioenergetic characteristic of tumor cells that may be affected
Fig. 3. Effects of cholesterol enrichment of rat liver mitochondrial membranes on their passive proton permeability. Mitochondrial proteins (40 mg) are incubated in the presence of 125 mg Sephadex-G10 beads coated (+) or not coated (C) with cholesterol in 1 ml of H-medium for 1 min at 0°C according to “Materials and Methods.” At the end of the incubation time, the beads are washed out by a centrifugation (9700 × g, 12 min, 4°C) through a 60% sucrose cushion on top of which mitochondria are collected. The mitochondrial pellet is washed by centrifugation at 9700 × g for 12 min at 4°C in H-medium, and the mitochondrial pellet is resuspended in 1 ml H-medium for the measure of passive proton permeability as indicated in “Materials and Methods.” The relative respiration rates (100 × V/Vmax) are plotted as a function of the added amounts of uncoupler FCCP to reach the rate V′. Experimental conditions are identical with those indicated in the legend of Fig. 2. Upon extrapolation of the line RLM corresponding to rat liver mitochondria (C) to a value of V/Vmax = 0, the FCCP amount obtained is equivalent, in absolute values, to the passive proton permeability. The same applies to RLM incubated in the presence of noncoated Sephadex beads (RLM+) and cholesterol-coated Sephadex beads (RLM + Chol). In order to subtract the effects of the Sephadex beads on mitochondrial membranes, the line RLM+ has been subtracted from the line RLM + Chol. The resulting line RLM Chol, which has the same vertical intersection as line RLM, gives the amount of FCCP corresponding to the passive proton permeability of cholesterol-enriched RLM. This experiment has been realized 3 times. One typical result is shown.

by a cholesterol increase in their membranes is mitochondrial phosphorylations.

Table 2 documents respiration measurements and estimated rates of ATP formation in RLM, ATM, and ETM. It may be noticed that respiratory control ratios (column 6) from ATM and ETM oxidizing succinate as substrate are, respectively, 61% and 69% that of RLM. These lower values are due to a decrease in the state 3 rates of respiration (column 2), since state 4 rates (column 1) are not significantly different from that of RLM. The ratio of the respiration rate when the uncoupler FCCP is present to the respiration rate when oligomycin is present (ratio FCCP/oligo, column 7) gives the maximal respiratory control values mitochondria should be able to reach. This amplitude is significantly 1.3-fold higher for ATM when compared to RLM, whereas it is equivalent in ETM and RLM. However, when the ratio FCCP:oligo is reported to the respiratory control ratio (column 8), ATM and ETM respectively display a 2.1- and a 1.5-fold higher ratio than for RLM. This means that both tumor mitochondria have the ability to reach maximal oxidation rates but do not oxidize succinate to such extreme values under our experimental conditions.

The table also gives ATP formation rates calculated on the basis of coupled respiration values (P/O ratios, column 5) and oligomycin-resistant respiration (column 3). P/O ratios for both types of tumor mitochondria are 89% that of RLM. The rates of mitochondrial ATP formation for both ATM and ETM are, respectively, 48.5% and 46.4% that for RLM, meaning that net ATP production in tumor mitochondria is about 2-fold lower than that of normal rat liver mitochondria. Assuming that the maximal oxidation capacities (represented in column 8) would be entirely devoted to ATP synthesis, RLM, ATM, and ETM would be at the origin of, respectively, 442.7 ± 15.0, 513.9 ± 17.9, and 339.0 ± 2.1 nmol ATP/min·mg protein. Under these theoretical circumstances, ATM would synthesize more ATP than RLM.

Presence and Role of Cytosolic CK Isozymes in Highly Glycolytic Tumor Cells. Another aspect of the deviant bioenergetics of the two glycolytic tumor cell lines resides in the appearance of high amounts of cytosolic CK.

Table 3 shows the activities of marker enzymes and of CK, the distribution of which is different in the Ehrlich and AS30-D tumor lines studied. In Ehrlich cells, CK is present mostly in the cytosolic fraction (with the highest lactate dehydrogenase activity) and in the postmitochondrial supernatant, representing, respectively, 70% and 22% of the total CK activity. The remaining 8% activity is retrieved in the other cellular fractions. Conversely, in AS30-D cells 66% of the total CK activity is present mostly in the postmitochondrial supernatant that contain cytoskeletal structures, small membrane particles, etc., and 13% of the activity is found in the nuclei plus heavy particle fraction. Fifteen % of the total CK activity is found in the cytosol. In both tumor cell lines, little if any CK activity is found in the mitochondria (fraction with the highest citrate synthase activity) or the mitoplast fraction.

The different CK isozymes have been separated and identified by a cellulose acetate electrophoresis (Fig. 4). When compared with the migration of marker enzymes (Fig. 4, Lane 1), AS30-D cells contain both BB- and MM-like activities in their cytoplasmic fraction (Fig. 4, Lane 2) and MM-like activity in their heavy particle fraction (Fig. 4, Lane 3). Ehrlich cells contain only BB-like activity, which is visualized solely in the cytosol (Fig. 4, Lane 5). In both cell lines no mitochondrial CK activity (m1 or m2) has been detected.

MM-CK activity is not present in hepatocytes. The unusual presence of cytosolic CK isozymes, especially in AS30-D
When cells are treated with 10 mM β-GPA, their growth rate is almost 2-fold lower than that of the controls. Moreover, the plateau is reached earlier on the fourth day of culture, when 7.5 x 10^5 living cells/ml are present, representing about 57% of the controls at the same day of culture. Therefore, CK isozymes are an indispensable element for tumor cell growth.

Cell viability at the exponential growth phase is 74.0 ± 1.9%, 83.1 ± 1.8%, and 86.0 ± 2.6% for, respectively, β-GPA-treated, creatine-treated, and control cells.

**DISCUSSION**

The data presented in this report show for the first time that the higher cholesterol content of mitochondrial membranes in highly glycolytic tumor cells is related to low passive proton permeability. They also show the presence of unusually high levels of cytosolic CK isozymes, the activity of which is not fortuitous but necessary for an efficient tumor cell growth.

Cholesterol levels are systematically high in each tumor cell line examined to date. In experimental hepatomas, the negative feedback control due to cholesterol on the key enzyme HMG-CoA reductase is lost (3); consequently, cholesterol progressively accumulates in cellular membranes. Metastatic cells have a lower level of sterol carrier protein 2 than do the original parental cells: the higher their malignancy, the lower their ability to produce or maintain adequate levels of sterol carrier protein 2 (4). For Lyons et al. (5), the low sterol carrier protein 2 levels in AS30-D hepatoma peroxysomes are directly related to a cholesterol accumulation in the cells. Each couple of carbon atoms contained in the cholesterol molecule takes its origin from acetyl-coenzyme A. This latter comes from mitochondrial citrate that is preferentially exported from tumor mitochondria and does not enter the truncated tumor Krebs cycle (24). We

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**Table 3** Marker enzyme and creatine kinase activity distribution in subfractions of Ehrlich and AS30-D cells

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>Cells</th>
<th>Lactate dehydrogenase</th>
<th>Citrate synthase</th>
<th>Creatine kinase</th>
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<tr>
<td>S2 (cytosol)</td>
<td>E</td>
<td>80%</td>
<td>10%</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>60%</td>
<td>6%</td>
<td>15%</td>
</tr>
<tr>
<td>P4 (nuclei + heavy particles)</td>
<td>E</td>
<td>2%</td>
<td>9.2%</td>
<td>5%</td>
</tr>
<tr>
<td>P6 (mitochondria)</td>
<td>E</td>
<td>4.5%</td>
<td>83.7%</td>
<td>3.4%</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>2.3%</td>
<td>64.0%</td>
<td>7.0%</td>
</tr>
<tr>
<td>S5 + S5' + S6</td>
<td>E</td>
<td>ND</td>
<td>ND</td>
<td>22%</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>ND</td>
<td>ND</td>
<td>66%</td>
</tr>
<tr>
<td>P8 (mitoplasts)</td>
<td>E</td>
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<td>0.7%</td>
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<tr>
<td></td>
<td>A</td>
<td>ND</td>
<td>ND</td>
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</tr>
</tbody>
</table>

*E. Ehrlich cells; A, AS30-D cells; ND, not determined.

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Fig. 4. Cellulose acetate electrophoresis of cytosolic fractions from Ehrlich and heavy particle fractions from AS30-D cells. Cell fractions are prepared according to the procedure described in “Materials and Methods.” Samples (containing 2–10 mU of CK in a 4–12 µl volume) are deposited on a cellulose acetate sheet. Electrophoretic migration is performed in a buffer containing 10 mM barbital, 60 mM sodic barbital, 60 mM Tris, 1 mM EDTA, 1 mM β-mercaptoethanol (pH 8.8), under an intensity of 6.5 mA for 40 min at 4°C. Activities are revealed by laying on top of the sheet a 1.2% agarose gel containing 70 mM nitro blue tetrazolium. The gel has been previously soaked in 0.1 M triethanolamine (pH 7.0), 20 mM glucose, 10 mM magnesium acetate, 1 mM ADP, 0.6 mM NADP, 10 mM AMP (to inhibit adenylate kinase), 35 mM phosphocreatine, 80 µM N-methylphenazonium methosulfate, 5.6 units hexokinase, and 2.8 glucose-6-phosphate dehydrogenase. The sandwich is incubated for 10 min at 37°C. Lanes 1–3, AS30-D. Lane 1, CK isozyme markers. The BB form migrates toward the positive pole. Lane 2, cytoplasmic fraction. Lane 3, heavy cellular particles. Lanes 4–7, Ehrlich. Lane 4, P4 fraction (heavy particles); Lane 5, S2 fraction (cytosol); Lane 6, P6 fraction (mitochondria); Lane 7, CK isozyme markers.

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Fig. 5. Growth curves for AS30-D cells treated with β-GPA (●) or with creatine (○) as compared to controls (O). Cells are incubated in the Dulbecco’s modified Eagle’s medium as indicated in “Materials and Methods.” Treated cells receive sterile 10 mM β-GPA or sterile 20 mM creatine, both in Dulbecco’s modified Eagle’s medium. Cell count and viability (represented by the ratio 100 x living cells/total cells) is done by trypan blue exclusion. Points, results of 8 experiments for β-GPA treatment and of 4 experiments for creatine treatment and controls; bars, SD.
have recently shown that mitochondria from Ehrlich and AS30-D glycolytic tumor cells decarboxylate pyruvate in a non-oxidative manner at a high rate to form high amounts of acetoin, an unusual compound for mammalian cells (8). When added to tumor mitochondria respiring on malate plus glutamate, acetoin is able to almost double the quantity of citrate produced, which, in turn, increases the level of synthesized cholesterol (9). All of these features explain that tumor cells contain a higher cholesterol level than their normal equivalent. This product may accumulate in every type of membrane and, in particular, in mitochondrial membranes. Data from this report show a 12.4- and a 2.3-fold higher cholesterol level, respectively, in ETM and ATM than in RLM, mainly due to a corresponding increase in the nonesterified cholesterol level, mostly in outer and inner mitochondrial membranes. Such a cholesterol content increase contributes to important biophysical property changes and, in particular, rigidification (25) that may have a noticeable effect on the passive proton leak of the inner mitochondrial membranes. Our data show that a 12.4- and a 2.3-fold higher cholesterol level in ETM and ATM, respectively, as compared to RLM is correlated to a 4- and a 1.4-fold lower proton passive permeability in ETM and ATM as compared to RLM. To show that this correlation is effective, we have enriched 3.5-fold the cholesterol content of normal RLM. As a consequence, we observed a 2-fold decrease in their passive proton permeability. Mitochondrial cholesterol content and passive proton permeability may be related according to a nonlinear relationship (not shown).

The H⁺ permeability of the membrane and thus the proton current flowing around the chemiosmotic proton circuit is one of the different rate-limiting steps controlling respiration (23). If we refer to an electrical circuit model, proton leakage will tend to decrease by increasing proton conductance. This leakage will decrease the thermodynamic ability to synthesize ATP. Since it is difficult to estimate the leakage current during ATP synthesis, we have extrapolated our calculations to the conditions for which proton leakage is zero. The FCCP values obtained under these circumstances allow us to estimate a proton leakage flow that is the highest in circumstances where ΔΨ is the highest. Calculation of this passive proton flow (JH⁺) takes into account the sensitivity of mitochondrial to the uncoupler, itself related to the partition coefficient of FCCP on the membranes, and a theoretical stoichiometry H⁺/O of 6 for succinate, which is consistent with the ratio H⁺/ATP of 3 (26). It has previously been shown that proton leakage across the inner membrane of mammalian mitochondria is significant and that its rate increases disproportionately with increased mitochondrial membrane potential ΔΨ (27). In the absence of oxidative phosphorylation and transport of other ions, all the protons pumped by the respiratory chain out of the mitochondria reenter it through the proton leak (28). In vitro and under our experimental conditions, the low proton leakage in cholesterol-enriched AS30-D tumor mitochondrial membranes slightly enhances the thermodynamic efficiency to make ATP; however, these mitochondria never reach the rate of ATP synthesis RLM display. This important feature may be explained in different ways. First, maximal state 3 rate of respiration is never reached, ADP and Pₐ being limiting at the level of phosphorylation sites in tumor mitochondria (29). This is probably due to the fact that in rapidly growing tumors the number of mitochondria is reduced by 50%: these organelles become weaker competitors for ADP and Pₐ than the already increased glycolysis. Second, the fluidity required for the respiratory chain to function properly is disturbed by membrane cholesterol enrichment. Finally, latent ATPase activity increases 2- to 3-fold as a function of the extent of cholesterol enrichment (30).

The reduction of the rate of ATP production from oxidative phosphorylation in tumor mitochondria renders essential the production of ATP by the glycolytic pathway. Recently, Arora and Pedersen (10) have shown that a hexokinase linked to the outer membrane of tumor mitochondria has a preferential access to mitochondrial ATP. It thus feeds glycolysis through glucose-6-phosphate and is at the origin of 60% of the cellular ATP amount. This poses the problem of the means for an efficient energy distribution in tumor cells, where most of the energy is devoted to cell division.

Another feature of these cells is the expression of CK isozymes. Normal liver creatine kinase activity, if present, is very low since it represents less than 0.3% of total muscle CK activity and is due to the BB isofrom. Both Ehrlich cells and AS30-D hepatoma cells have a higher BB-CK activity (fetal form). A colored spot is visualized on a cellulose acetate electrophoresis at the position where MM-CK activity is usually found (Fig. 4). Since adenylylate kinase migrates at the same location under identical conditions, we cannot exclude that part of this activity could be due to a residual adenylylate kinase activity (even though AMP is used to inhibit it). Thus, the AS30-D line could enter the category of rat hepatomas studied by Shatton et al. (11), where the simultaneous presence of both BB and MM forms has been found, in particular in the differentiated lines of the Morris series and Novikoff hepatoma (11). However, other hepatoma lines contain only the BB isofrom. The presence of most of the CK activity in the heavy particle fractions of AS30-D cells (see Table 3) reinforces our hypothesis that two isofroms exist in these cells. Moreover, electron micrographs of these cells show the presence of many cytoskeleton filaments that should be involved in cell division processes. CK has been found to be compartmentalized, in association with subcellular structures, suggesting that the communication and transfer of energy-rich compounds between ATP-generating sites (mitochondria and glycolysis) and ATP-utilizing sites are facilitated via PCr/Cr systems (31). A Cr circuit model has been proposed (32) in which the Cr produced by oxidative phosphorylation via mitochondrial ATP enters the cytosolic PCr/Cr/ATP/ADP equilibrium. The latter is governed by the cytosolic CK functionally coupled to glycolysis. Thus it may be possible to consider a functional association, in cancer cells, between a soluble CK isofrom and a CK isofrom linked to cytoplasmic structures, which could be involved in an efficient energy pathway distribution. Our results confirm the presence of only the fetal BB isofrom of CK in the Ehrlich cells as found by Shatton et al. (11), mainly encountered in the cytosolic fraction and thus in a soluble form.

When these two isofroms are present, they are probably the result of a genetic derepression due to a misprogramming of proteosynthesis. It has recently been demonstrated that the BB isofrom is produced by murine hybridomas but not by parental cells (33). It has recently been suggested that a BB isofrom could partially be linked to the outer face of the inner mitochondrial membrane of Ehrlich tumor cells (34). However, we were unable to reproduce this result, the BB isofrom we found being exclusively cytosolic. No mitochondrial CK isofrom seems therefore to have been identified to date.

Evidence of the role of cytosolic CK in efficient energy distribution for tumor cell divisions is also provided by our experiments with the creatine analogue β-GPA, which is much slowly
metabolized than creatine (35). Creatine (and thus CK) involvement is clearly demonstrated by the fact that β-GPA significantly slows AS30-D tumor cell growth in culture conditions and decreases cell viability. A similar in vitro Ehrlich ascites growth reduction has been found recently after tumor-bearing mice were fed β-GPA (36). Decreased PCr levels together with increased levels of inorganic phosphate have already been found in cancer cells (37) and have been considered to be due to increased glycolysis (37) and to a higher turnover rate of PCr mobilized by CK.

The presence of a hexokinase linked to the outer mitochondrial membrane of tumor cells may form, besides cytosolic CK isozymes, groups of energetic distribution, the significance of which may differ markedly from that of normal cells. The presence of both linked hexokinase and cytosolic CK make possible a competition for respiratory ATP, hexokinase feeding glycolysis (10), and recycling of cytoplasmic ATP into PCr by CK (38).

It has been shown that β-GPA supplementation is not toxic for normal tissues (39). Since it significantly slows tumor cell growth and decreases tumor cell viability, β-GPA may represent an interesting value for adjuvant cancer therapy.

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REFERENCES

Low Mitochondrial Proton Leak Due to High Membrane Cholesterol Content and Cytosolic Creatine Kinase as Two Features of the Deviant Bioenergetics of Ehrlich and AS30-D Tumor Cells

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