Aberrant Mitochondria in Two Human Colon Carcinoma Cell Lines

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ABSTRACT

Electron micrographs of CCL237 and FET cells (two slowly growing, differentiated human colon carcinoma lines) revealed enlarged mitochondria with few cristae. Polarographic measurement of respiratory activity in mitochondria isolated from these cell lines was compared to that of CV-1 cells (a normal monkey kidney epithelial line) and MIP101 cells (another human colon carcinoma line), both of which have mitochondria with a "normal" appearance. The respiratory control ratios of CCL237 and FET mitochondria were found to be considerably lower than those of CV-1 and MIP101 mitochondria (approximately 3 as compared to >10, respectively), indicating that in CCL237 and FET mitochondria the processes of substrate oxidation and phosphorylation of ADP are only loosely coupled. In intact cells, differences in radiolabeled tetraphenylphosphonium uptake showed that the mitochondrial membrane potential in CCL237 and FET cells was less than that in CV-1 and MIP101 cells, and that nigericin failed to hyperpolarize the mitochondria of CCL237 and FET cells. In addition, FET mitochondria exhibited significantly lower ADP-stimulated and uncoupled respiratory rates than mitochondria isolated from the other cell types, indicating that in the former, the capacity for oxidative phosphorylation is somehow impaired. Selective toxicity of FET cells was obtained by treatment with 2-deoxyglucose, an inhibitor of glycolysis, suggesting the possibility of exploiting the phenotype of impaired oxidative metabolism for chemotherapy.

INTRODUCTION

Numerous pathological conditions are characterized by mitochondria that display unusual morphological features (for review, see Ref. 1). Various abnormalities have been described with regard to the size and shape of this organelle, the number and organization of its cristae, and the absence or presence of "dense-granule" inclusions in the matrix. The tissues displaying these unusual mitochondria include the brain, liver, cardiac and skeletal muscle, kidney, and adrenal cortex. In most cases, these ultrastructural peculiarities are associated with a particular biochemical alteration, such as a defect in substrate oxidation or ion transport systems, or a deficiency in enzyme levels or cytochrome content. Whether these mitochondrial abnormalities are a cause or consequence of a given pathological state is not always easily determined. However, since mitochondrial occupancy 15 to 50% of the total cytoplasmic volume of most animal cells and participate in more metabolic functions than any other organelles, it is apparent that their proper functioning is essential to maintaining a normal cellular metabolism. The fluorescent mitochondrial stain rhodamine 123 has been used extensively in living cells to visualize mitochondrial morphology and monitor changes in mitochondrial function (2-4). Over 200 different cell lines/types were screened in this laboratory for rhodamine 123 uptake. While the mitochondria of most carcinoma cells exhibit increased uptake and retention of the dye (5, 6), two slowly growing, differentiated human colon carcinoma lines (CCL237 and FET) displayed unusually low levels of rhodamine 123 uptake. The main objective of the present study was to determine the basis for this phenomenon.

Electron micrographs of FET and CCL237 cells revealed mitochondria with striking morphological features; the organelles were enlarged and contained few cristae. Through measurement of various parameters of oxidative metabolism, including respiratory function in isolated mitochondria and TPP uptake under various conditions in whole cells, it has been determined that these ultrastructural abnormalities are associated with some biochemical impairment in respiratory function. The possibility of exploiting this phenotype for chemotherapy was investigated.

MATERIALS AND METHODS

Cell Cultures. CV-1 cells, a normal monkey kidney epithelial line, were grown in DME medium (GIBCO) supplemented with 5% calf serum (Hazelton). Human colon carcinoma cell lines FET, obtained from Dr. M. Brittain (7), and MIP101, obtained from Dr. D. Zamcheck (8), were grown in equal volumes of DME and RPMI 1640 (GIBCO) supplemented with 5% calf serum and 5% Nu serum (Collaborative Research). CCL237, another human colon carcinoma cell line, was grown in RPMI 1640 supplemented with 10% fetal calf serum (Hazelton).

Fluorescence Microscopy. Cells grown on glass coverslips were exposed to rhodamine 123 (50 µg/ml for 30 min; Eastman Organic Chemicals, Rochester, NY), washed, and mounted on silicon rubber chambers containing dye-free culture medium as previously described (2). Fluorescence of rhodamine 123 was observed as previously described (2), using a Zeiss Photomicroscope III equipped with epifluorescence optics.

Electron Microscopy. Cell monolayers were fixed in situ for 1 h at room temperature with 2.5% glutaraldehyde and postfixed for 1 h at 4°C in 1% OsO4 with 1% potassium ferricyanide. After washing, monolayers were dehydrated in graded alcohols and embedded in Epon 812, and ultrathin sections were cut parallel to the surface by a Sorvall MT2 microtome. After staining in uranyl acetate and lead citrate, grids were examined by a Philips 300 electron microscope. Electron microscopy was performed by Elizabeth Beaumont.

Isolation of Mitochondria. Mitochondria were isolated from cells in culture as described previously (9). Typically, 5 x 10⁶ cells were harvested in culture medium, pelleted, and washed once with homogenization buffer (250 mM sucrose:1 mM Tris-HCl:1 mM EDTA:1 mg/ml of bovine serum albumin, pH 7.4). The cells were resuspended to a volume of 7 ml and homogenized in a Dounce tissue grinder until at least 95% of the cells were disrupted (approximately 125 strokes). The homogenate was centrifuged at 800 × g for 10 min at 4°C. The supernatant was removed and saved, and the pellet was resuspended and centrifuged again at 800 × g for 10 min. The supernatants were then pooled and centrifuged at 9400 × g (10 min, 4°C); the pellet was resuspended, centrifuged again at 9400 × g, and finally resuspended to a volume of about 5 mg/ml. Protein was determined by the Bio-Rad protein assay.

Respiration. Oxygen consumption was measured polarographically with a Clark electrode in a 1-ml water-jacketed chamber maintained at 30°C (10). The basic respiratory assay medium consisted of 225 mM sucrose, 10 mM KCl, 1 mM EDTA, 10 mM K₂HPO₄-KH₂PO₄, 5 mM MgCl₂, 10 mM Tris-HCl, and 1 mg/ml of bovine serum albumin, pH 7.4, to which additions were made in the following order: mitochondria (0.18 to 0.27 mg of protein); rotenone (2 µg/ml); succinate (10 mM); ADP (120 nmol); and 2,4-dinitrophenol (80 µM). The ADP-stimulated
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respiratory rate was defined as the rate after ADP minus the rate before ADP, while the uncoupled respiratory rate was defined as the rate in the presence of 2,4-dinitrophenol. The RCR was calculated by dividing the rate of respiration obtained after ADP addition (State 3) by the rate of respiration when ADP is limiting (State 4).

Uptake of TPP. Uptake of radiolabeled TPP was monitored according to the procedure of Davis et al. (11). Briefly, cells were grown in their appropriate medium on 12-mm glass coverslips (Bradford Scientific Co., Epping, NH) with a seeding density of approximately 5 x 10⁴ cells per coverslip. Cells were incubated with [3H]tetraphenylphosphonium chloride (specific activity, 4.3 Ci/mol; final concentration, 1 to 3 nM; Dupont-New England Nuclear, Boston, MA) in either low K⁺ medium ([137 mM NaCl:3.6 mM KCl:0.5 mM MgCl₂:1.8 mM CaCl₂:4 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid:1 mg/ml of dextrose:1% modified Eagle’s medium-amino acid solution (100x; GIBCO), pH 7.2) or high K⁺ medium ([137 mM KCl:3.6 mM NaCl]other components same as low K⁺ medium). At specific time points, coverslips were removed from the medium and rinsed several times with phosphate-buffered saline. Radioactivity was determined by liquid scintillation counting. The uptake ratio was calculated by dividing the total cellular uptake in cpm by the cpm in a volume of external medium equal to the volume of cells. Estimates of cell volume were obtained by centrifugation of cell suspensions in a graduated tube. Results presented in Figs. 3 and 4 are the mean of 2 to 4 separate determinations.

Cytotoxicity Assay. Cells were seeded at 5 x 10⁵ cells/60-mm dish and incubated at 37°C in 5% CO₂. 2-Deoxyglucose (2.5 mM) was applied the same day and, at specified intervals during continuous drug exposure, cells were trypsinized and counted by hemocytometer. The exclusion of trypan blue was used as an indicator of live cells.

RESULTS

Rhodamine 123 Staining. Living cells stained with rhodamine 123 (Fig. 1) show that the mitochondria of CV-1 cells (normal epithelial) take up much more of the dye and fluorescence much more brightly than those of CCL237 and FET cells (2 human colon carcinomas). This is unusual since most carcinoma cells exhibit an increased uptake and prolonged retention of rhodamine 123 and other lipophilic cations compared to CV-1 cells (3, 5, 9, 11).

Ultrastructure. Ultrathin sections were cut from embedded glutaraldehyde-fixed monolayers of cells. Electron micrographs (Fig. 2) of these sections show that, while CV-1 and MIP101 cells contain mitochondria with no unusual structural characteristics, FET and CCL237 cells contain enlarged mitochondria with few cristae.

Mitochondrial Respiration. Polarographic measurements of respiratory activity in mitochondria isolated from FET and CCL237 cells were compared to those from CV-1 and MIP101 cells, both of which contain “normal” looking mitochondria. The RCRs of mitochondria isolated from FET and CCL237 cells are significantly lower than those of mitochondria isolated from CV-1 and MIP101 cells (Table 1). The RCR is a criterion used to assess the extent of coupling between mitochondrial respiration and ATP synthesis. While RCR values greater than 10 are indicative of well-coupled mitochondria, much lower values, resulting from an increase in State 4 respiration, are associated with an abnormal leak of protons through the inner membrane in the absence of ADP and are indicative of a “loose coupling” of the processes of substrate oxidation and ATP synthesis. In addition, Table 1 shows that FET mitochondria exhibit much lower ADP-stimulated and uncoupled respiratory rates than mitochondria isolated from the other cell types. This suggests that, in FET cells, the capacity for mitochondrial respiration is somehow impaired.

TPP Uptake. The permeant cationic compound TPP was used to monitor membrane potential in whole cells. Since the plasma membrane potential of epithelial cells is thought to be primarily a K⁺ diffusion potential (12), a medium high in K⁺ concentration should depolarize the plasma membrane, and therefore differences in TPP accumulation may be attributed solely to the mitochondria membrane potential. Fig. 3 depicts approximately 3- to 5-fold greater accumulation of TPP in high K⁺ medium in MIP101 and CV-1 cells than in either FET or CCL237 cells. This suggests that the mitochondrial membrane...
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Fig. 2. Electron micrographs of FET (A) (x 15,228), CCL237 (B) (x 37,989), MIP101 (C) (x 14,555), and CV-1 (D) (x 12,820).

Table 1 Respiratory function of mitochondria isolated from cells grown in culture

<table>
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<tr>
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<th>Mitochondrial respiratory rate (nanoatoms of oxygen/min/mg)</th>
<th>State 3</th>
<th>ADP-stimulated</th>
<th>State 4</th>
<th>Uncoupled</th>
<th>RCR</th>
</tr>
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<tr>
<td>CV-1</td>
<td>106.5 ± 4.7</td>
<td>70.6 ± 3.6</td>
<td>3.8 ± 0.8</td>
<td>107.8 ± 3.5</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>MIP101</td>
<td>88.6 ± 27.7</td>
<td>57.6 ± 16.9</td>
<td>2.8 ± 0.4</td>
<td>90.5 ± 17.0</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>CCL237</td>
<td>140.7 ± 31.7</td>
<td>70.9 ± 16.3</td>
<td>51.4 ± 4.6</td>
<td>103.5 ± 19.3</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>FET</td>
<td>60.6 ± 12.3</td>
<td>21.7 ± 1.0</td>
<td>23.5 ± 8.7</td>
<td>32.5 ± 5.5</td>
<td>2.9</td>
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* Mean ± SE of at least 3 separate determinations.

potential in FET and CCL 237 cells is significantly less than that found in MIP101 or CV-1 cells.

We also measured TPP uptake in low K⁺ medium, a condition which more closely resembles the normal cellular environment than does high K⁺ medium. Fig. 4 shows that, in the presence of 5 μg/ml of nigericin, a K⁺/H⁺ exchange ionophore which dissipates the pH gradient across the mitochondrial inner membrane while compensatorily increasing the mitochondrial membrane potential, there is a significant increase in TPP uptake over control values in both CV-1 and MIP101 cells, yet no significant effect on TPP uptake in FET and CCL237 cells.
about 10% of the original treated population), whereas the total number of control cells after 10 days of growth was $5.95 \times 10^4$, representing an approximate 6-fold increase in cell number over the original untreated population. In contrast, 10 days of constant exposure to 2.5 mM 2-deoxyglucose resulted in a 60-fold increase in MIP101 cells over the original treated population, while the number of control cells increased by 120-fold, a 50% decrease in the rate of cell growth attributable to 2-deoxyglucose. This compound also inhibited the rate of cell growth of CV-1 cells, while having a minimal effect on CCL237 cells (data not shown).

**DISCUSSION**

Certain unusual morphological features have been characterized previously for tumor mitochondria in situ. Hruban et al. (13) noted that some tumor cell lines exhibit differences in the size of their mitochondria relative to normal controls; mitochondria from rapidly growing tumors tended to be smaller with fewer cristae, whereas those from slowly growing tumors were larger with characteristics more closely resembling those of normal cells. Although both FET and CCL237 are slowly growing, it is apparent that the mitochondria of these cell lines do not resemble normal mitochondria. Isolated tumor mitochondria have been shown to display abnormal shapes, unusual cristae, helical structures, or inclusions (14-16; see Ref. 17 for review). Most of these studies, however, involved chemically induced hepatomas. CCL237 and FET may be the first human colon carcinoma cell lines demonstrated to have aberrant mitochondrial morphology.

We investigated whether these ultrastructural abnormalities are associated with impairments in mitochondrial respiratory function. Polarographic measurements of respiratory activity in mitochondria isolated from FET and CCL237 cells were compared to values obtained for mitochondria isolated from a normal epithelial cell type (CV-1) and another colon carcinoma line where this organelle displayed no unusual morphological characteristics (MIP101). As Table 1 illustrates, mitochondria isolated from FET cells exhibit significantly lower ADP-stimulated and uncoupled respiratory rates when compared to mitochondria from other cell types. These data appear to be indicative of an impaired capacity for mitochondrial oxidative phosphorylation, a phenomenon first described by Warburg more than 50 yr ago (18). In addition, the low RCR values exhibited by CCL237 and FET mitochondria indicate that, in these cells, the processes of substrate oxidation and phosphorylation of ADP are only loosely coupled. Interestingly, this is not the first time that a pathological condition has been found to be associated with this particular mitochondrial abnormality.
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In 1962 Luft et al. (19) described the case of a 35-yr-old woman with severe hyperthermia and muscle myopathy. Electron microscopic examination of a muscle biopsy revealed abnormally large mitochondria with tubular cristae and rod-like inclusions. Measurements of mitochondrial respiratory function indicated normal State 3 respiratory rates (which occur in the presence of substrate, P_n, and ADP), yet abnormally high rates of State 4 respiration (which occur when ADP is limiting). Luft et al. attributed the patient's symptoms to the "loose coupling" of muscle mitochondria.

We then considered whether these differences in bioenergetic function would be expressed by mitochondria in situ. Fig. 3 illustrates that the mitochondrial membrane potential in CCL237 and FET cells is much lower than that found in CV-1 and MIP101 cells. This accounts for the unusually low uptake of rhodamine 123 observed in CCL237 and FET cell lines. In addition, we show that, while nigericin hyperpolarizes the mitochondria of CV-1 and MIP101 cells, there is no such hyperpolarization in either CCL237 or FET cell lines (Fig. 4). This can be explained by the fact that, while nigericin mediates the electroneutral exchange of K+ ions for protons, hyperpolarization can occur only at the expense of an existing pH gradient (as is the case for CV-1 and MIP101). However, if mitochondria have an impaired respiratory capacity and/or are only loosely coupled (as is the case for CCL237 and FET cells), there is no substantial pH gradient and therefore no hyperpolarization upon addition of nigericin. These results are significant in that they confirm those obtained for isolated mitochondria and establish the fact that the low RCR values of isolated FET and CCL237 mitochondria do not result from damage to the organelles during the course of isolation.

Mitochondrial oxidative phosphorylation and glycolysis are the two major pathways for cellular ATP production. It may therefore be possible to obtain selective killing of those cells exhibiting an impairment in oxidative metabolism by blocking their alternative energy supply. We tested the effect of 2-deoxyglucose, an inhibitor of glycolysis, on the growth of cultured FET cells (with aberrant mitochondria) and MIP101 cells (with normal mitochondria). While the assay medium contained glucose as its primary fuel source, it also contained oxidizable substrates such as pyruvate, lipids, and various amino acids. As shown in Fig. 5, 10 days of constant exposure to 2.5 mM 2-deoxyglucose exert a potent cytotoxic effect on FET cells, whereas in MIP101 cells, it merely inhibits the rate of cell growth. Interestingly, 2-deoxyglucose was found not to be cytotoxic to CCL237 cells (data not shown), suggesting that it is not the degree of coupling but rather the capacity for mitochondrial respiration that is the determining factor for selective killing by this compound.

The incidence of colon cancer in the United States remains high. There are 160,000 cases reported annually, with 60,000 deaths per year being attributed to this disease. Most traditional forms of treatment for this disease have been aimed at inhibition of DNA metabolism. We appear to have identified a distinct class of human colon carcinomas that display impairments in mitochondrial bioenergetic function. The fact that selective cytotoxicity of FET cells was obtained with 2-deoxyglucose is important in that it suggests the possibility of exploiting this phenotype as a target for chemotherapy. The frequency with which an impairment in mitochondrial function occurs in human colon carcinomas and the efficacy of 2-deoxyglucose in the treatment of this class of cancer remain to be determined.

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REFERENCES

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