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Original

TCA Cycle Enzymes in Mitochondrial Cells (MitoCells)

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Culturing mitochondria outside of cells was once considered to be virtually impossible. We previously isolated mitochondrial cells (MitoCells) to establish stable cell lines from cybrids obtained by fusing mtDNA-less-HeLa cells with platelets (Nakano et al, 2003). MitoCells lack nuclei and have only a small amount of nuclear DNA, but these cells can maintain mitochondrial activity. However, energy-producing processes of MitoCells remain poorly understood. Herein, we cultured MitoCells under anaerobic conditions and analyzed enzymes and activities of the tricarboxylic acid (TCA) cycle. MitoCells survived under anaerobic culture conditions for over 30 days, while cybrids disappeared within a week. Western blot analysis confirmed the absence of pyruvate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase in MitoCells. The citrate synthase activity in MitoCells was also lost. These results suggest that MitoCells produce energy employing an anaerobic metabolic pathway. Considering that MitoCells were originally derived from human cells, our results suggest that human nuclear DNA may harbor an anaerobic energy production pathway. Clinically, the hypoxia associated with cancer is attributable to hypoxic tumors. Progress in understanding the anaerobic metabolic pathway in MitoCells may allow elucidation of the anaerobic metabolic pathway in highly malignant tumors and facilitate the development of anticancer therapies.

Key Words: mitochondrial cells, tricarboxylic acid cycle, aerobic metabolism, anaerobic metabolism, hypoxic tumor

Introduction

It was long believed that mitochondria cannot be cultured outside of cells. Nakano et al, however, isolated a stable, novel line of mitochondrial cells designated MitoCells from cybrids obtained by fusing mtDNA-less-HeLa cells with platelets. MitoCells lack nuclei but maintain mitochondrial activity including mitochondrial membrane potential. These MitoCells grow continuously in culture despite the absence of nuclei and can thus be continuously propagated in culture. MitoCells, almost all of which contain only small amounts of DNA, grow continuously in culture[1]. Mitochondrial membrane potentials of MitoCells stained with Mito Tracker Red in our previous study confirmed that MitoCells are alive. Like other organisms, living MitoCells must produce adenosine triphosphate (ATP) and use ATP to maintain life. However, the mechanism by which MitoCells produce ATP remains unknown. There may be an unknown mechanism promoting the growth and maintenance of these cells, while the main metabolic pathway of eukaryotic cells is mitochondrial oxidative phosphorylation through the tricarboxylic acid (TCA) cycle. We, therefore, cultured MitoCells under anaerobic conditions and analyzed enzymes and activities of the TCA cycle in order to elucidate the biological characteristics of MitoCells.

Materials and Methods

We performed an anaerobic culture experiment on MitoCells and analyzed certain enzymes and activities of the TCA cycle in MitoCells to evaluate TCA cycle functions.

1. Anaerobic culture experiment

1) Cell cultures

MitoCells were obtained as previously described[1]

and cultured in Rosewell Park Memorial Institute medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin at 37℃ in a humidified gas mixture containing 8% CO_2. Cybrids were obtained as previously described[1] and cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.2 mM uridine, 2 mM glutamine and 1 mM sodium pyruvate at 37℃ in a humidified gas mixture containing 8% CO_2.

2) MitoCells under anaerobic culture conditions

MitoCells and cybrids were cultured using Dia anaerobic pack (O_2<0.1%, CO_2 21%, 37℃) in each medium for at least one month. The cells were observed by light microscopy.

2. Enzyme assays of the TCA cycle with Western blotting

1) Antibodies

The primary antibodies used for Western blotting were mouse monoclonal antibodies against tubulin (Clone Ab-4; Neomarkers, Fremont, CA, USA and Clone ab56676 Abcam, Tokyo, Japan), citrate synthase (Clone D3 G4; Abcam), malate dehydrogenase (Clone A-4; Abcam), pyruvate dehydrogenase beta subunit (Clone E-1; Abcam), isocitrate dehydrogenase 2 (Clone M01; Abnova, Taipei, Taiwan), and succinate dehydrogenase hydratase (Clone 2E3; Santa Cruz Biotechnology, Santa Cruz, CA, USA). At use, each antibody was diluted 1:500. The secondary antibodies were anti-mouse IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:5,000.

2) Protein extraction

Cybrids and MitoCells were centrifuged for 10 min at 1,000 rpm. The pellets were then washed with Hank's buffer and centrifuged for 10 min at 1,000 rpm. Then, each suspension was homogenized by passage through a 26 gauge needle. After the homogenized samples had been washed with Hank's buffer and centrifuged for 1 min at 10,000 rpm, distilled water was added to the pellet. Protein concentrations were measured in aliquots of the samples. Sodium dodecyl sulfate (SDS) gel-loading buffer was added to the remaining samples which were then boiled for 5 min before loading on SDS gel for Western blotting.

3) Western blot analysis

Proteins resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide gels) were electroblotted onto nitrocellulose (Sigma-Aldrich, Saint Louis, MO, USA). Ten micrograms of protein were loaded into both cybrids and MitoCells. The blots were blocked with 1% casein blocking solution (Bio-Rad) for 1h at 4℃ and then incubated with primary antibodies for 1h at 4℃.

For colorimetric detection, washed blots were incubated with secondary antibody diluted in Tris Buffered Saline with Tween (TBST, Bio-Rad) for 1h at room temperature. The blots were then washed in TBST and a color reaction was elicited with NBT/BCIP substrate tablets (Roche, Indianapolis, IN, USA). Tubulin was electroblotted as an inner control.

3. Enzyme activities of TCA cycle and mitochondrial electron transport

1) Materials

The following samples were used for enzyme activity analysis. The MitoCells (Mit) #1 line consists of MitoCells transformed from cybrids with a T9176C mitochondrial mutation. The T9176C mitochondrial mutation, located in the ATPase 6 gene of mitochondrial DNA, is pathogenic for Leigh syndrome. Mit #1 was derived from one sibling in a family with Leigh syndrome harboring the T9176C mitochondrial mutation. The Mit #2 line consists of MitoCells without the mutation transformed from control cybrids. Cybrid cell line (Cyb) #1 (cybrids with T9176C mitochondrial mutation), as well as lines Cyb #2a and #2b (cybrids without the mutation), were used as controls for MitoCells. Cyb #2a and #2b underwent serial passages from the same cell line.

2) Miniaturized mitochondrial assays

Miniaturized assays were employed for the measurement of protein, complex II + III (succinate cytochrome c oxidoreductase), complex IV (cytochrome c oxidase) and citrate synthase (CS) activities using an iEMS reader MF (Labsystem Corp., Yokohama,

JAPAN). The procedure developed for the miniaturized assays for complexes II + III and IV is presented below. The CS assay was previously described in detail[2)3)].

3) Complex II + III assay (succinate: cytochrome c oxidoreductase)

The 25-μl diluted samples, which consisted of four different protein concentrations, were added to wells. Next, 175 μl of 71 mM potassium phosphate, pH 7.4, with 10.44 mg% NaCN and 2.84 μM rotenone, were added to each well. Then, 25 μl of 200 mM succinate in water were also added. The mixture was pre-incubated in the dark for 10 min at 37℃. The reaction was started by adding 25 μl of 1 mM cytochrome c (Sigma type IV). The rate of the cytochrome c increase at 550 nm was measured for 4 minutes at 37℃. For this calculation, the activity must be multiplied by a factor of 0.648 to correct for the length of the light path and microplate absorption. The coefficient of variation in complex II + III reproducibility (three assays were performed) was 7.3%.

4) Complex IV (cytochrome c oxidase) assay

A 175-μl quantity of 25 mM potassium phosphate, pH 7.0, was added to each well. The 25-μl diluted samples, with four different protein concentrations, were then added to the wells. After pre-incubation for 6 min at 37℃, the reaction was initiated by adding 25 μl of 25 mM reduced cytochrome c in 20 mM potassium phosphate, at pH 7.0. The decrease in absorption at 550 nm was measured for 120 seconds and maximally oxidized absorption was determined by adding potassium ferricyanide at completion. Then, 25 μl of 10 mg/ml dodecylmaltoside were added before pre-incubation. As Box Cox analysis indicated inhomogeneity of variance, all analyses were performed using the natural logarithmic transformation of the data. The coefficient of variation in complex IV reproducibility (three assays were performed) was 5.2%.

5) Citrate synthase (CS) assay

The assay utilized is a modification of the technique described by Haas et al[4)]. CS was assayed in Triton X 100 solubilized MitoCells or cybrid cells. The total working volume was 250 μl. Sample wells contained 125 μl of buffer (0.16 M Tris, pH 8.0, 2 μl of 10 mM fresh 5',5'-dithio-bis (2-nitrobenzoic acid (DTNB) in 0.1 M Tris, pH 8.3), 25 μl of diluted samples with four different protein concentrations, 25 μl of Triton ×100 0.8% in water, and 25 μl of acetyl coenzyme A (acetyl-CoA; 0.5 mM). The reaction was

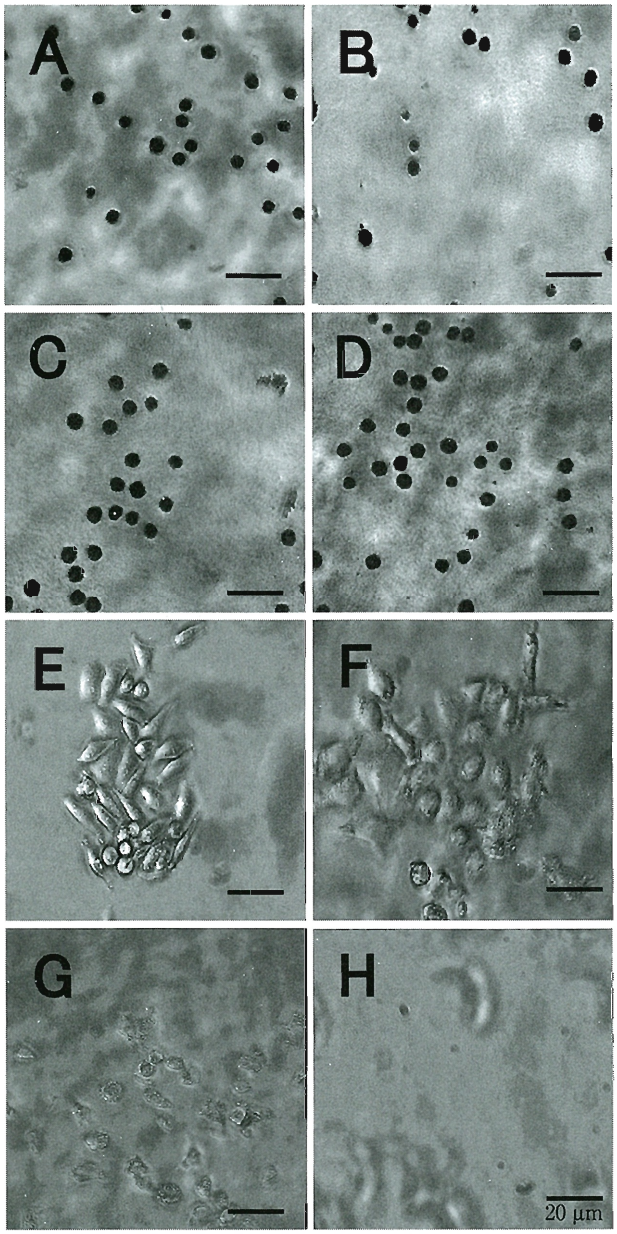

Fig. 1 Phase-contrast images of MitoCells and cybrids observed by light microscopy at ×200 magnification (Bar = 20 μm). These cells were cultured using Dia anaerobic pack (O_2<0.1%, CO_2 21%, 37℃) in each medium for at least 40 days.
MitoCells at day 1 (A), day 7 (B), day 14 (C) and day 40 (D).
Cybrids Day 1 (E), Cybrids Day 3 (F), Cybrids Day 7 (G) and Cybrids Day 14 (H).

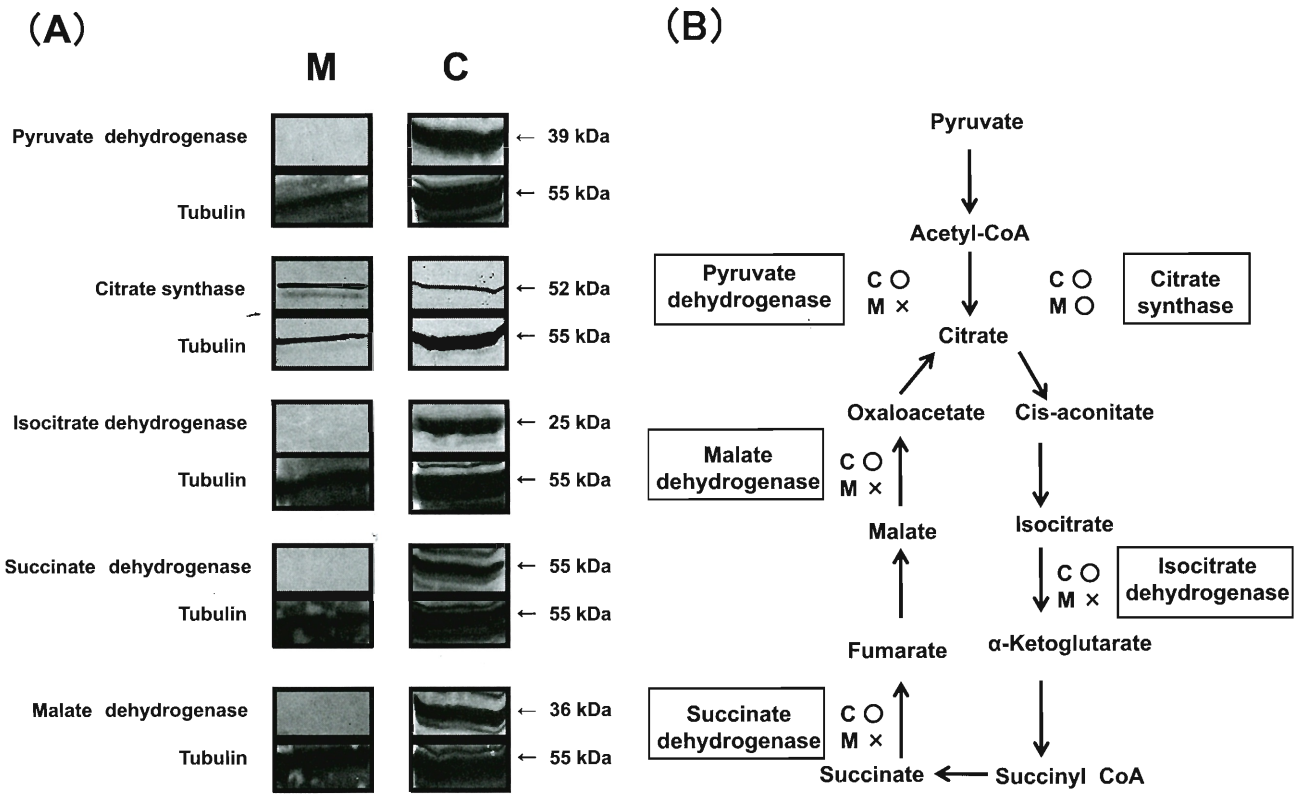

Fig. 2 Expressions of TCA cycle-related enzymes in total lysates of MitoCells (M) and cybrid cells (C). Panel (A) is representative immunoblots of pyruvate dehydrogenase (39 kDa), citrate synthase (52 kDa), isocitrate synthase (25 kDa), succinate dehydrogenase (55 kDa), and malate dehydrogenase (36 kDa). Panel (B) is a schematic diagram of preserved (○) and reduced (×) expression levels of these enzymes.

started after a 3 min pre-incubation at 30℃ by adding of 25 μl of freshly made oxaloacetate (5 mM in water). The increase in absorption of the 5-thio-2-nitrobenzoate ion at 412 nm was measured. The results of this assay are multiplied by a correction factor of 0.676 to correct for well dimensions and absorption. The coefficient of variation in CS reproducibility (four assays were performed) was 2.0%.

Results

1. MitoCells survive and grow under anaerobic conditions

We found that MitoCells survived without morphological changes under anaerobic culture conditions for more than 30 days: the round brown MitoCells were dispersed at the beginning of culture and their shape and distribution were maintained through 40 days (Fig. 1-A, B, C and D). On the other hand, cybrids had disappeared by day 14: cybrids were spindle-shaped and showed colony formation at the beginning of culture. On day 3, cybrids became swollen and the colonies collapsed. On day 7, cybrids became atrophic and their numbers were reduced. By day 14, cybrids had disappeared. (Fig. 1-E, F, G and H).

2. Western blot analyses of TCA cycle enzymes in MitoCells

To determine whether or not MitoCells use the TCA cycle, we performed Western blot analyses of various TCA cycle enzymes in MitoCells. We found that only citrate synthase was present in MitoCells, while malate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase were not. In the control cybrids, all of these enzymes were present (Fig. 2).

3. Mitochondrial enzyme assays with microplate-reader (Fig. 3)

Complex II + III activities of MitoCells (Mit#1, Mit#2) were 15.9 ± 0.07 and 15.8 ± 0.81 (nmol/min/mg of protein), while those of cybrids were 10.91 ± 0.23, 10.95 ± 0.62 and 18.7 ± 1.45 (Cyb#1, #2a, and

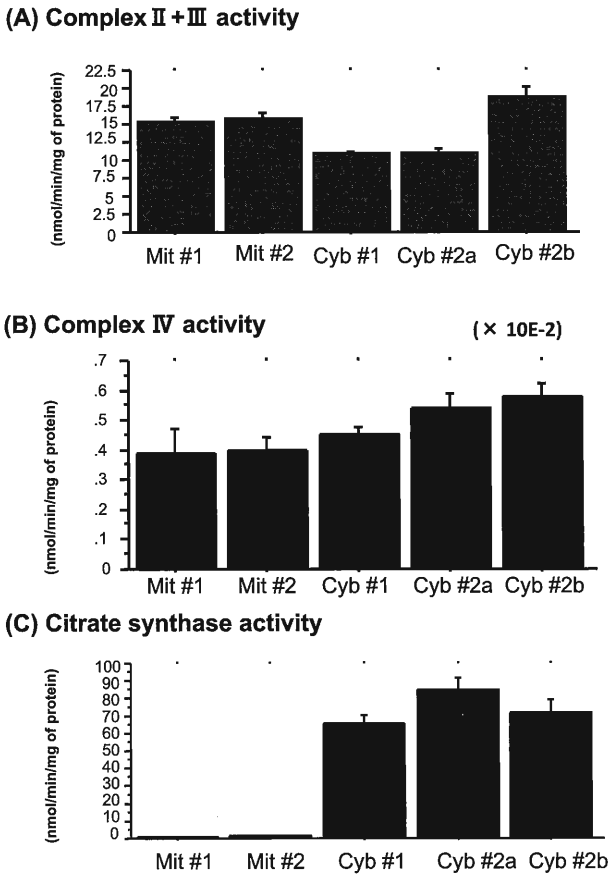

Fig. 3 Mitochondrial enzyme activities of MitoCells and Cybrid cells
Panel (A) indicates complex II+III enzyme activity. Panel (B) indicates complex IV enzyme activity. Panel (C) indicates citrate synthase enzyme activity.

#2b). The complex II + III activities of MitoCells were comparable to those of Cyb#1, #2a and #2b. The complex IV activities of MitoCells (Mit#1, #2) were $0.33 \times 10^{-2} \pm 0.08 \times 10^{-2}$ and $0.40 \times 10^{-2} \pm 0.06 \times 10^{-2}$, while those of cybrids (Cyb#1, #2a, and #2b) were $0.45 \times 10^{-2} \pm 0.026 \times 10^{-2}$, $0.54 \times 10^{-2} \pm 0.05 \times 10^{-2}$ and $0.58 \times 10^{-2} \pm 0.045 \times 10^{-2}$ (nmol/min/mg of protein). The complex IV activities of MitoCells were slightly lower than those of cybrids. The citrate synthase activities of MitoCells (Mit#1, #2) were 1.1 ± 0.08 and 1.7 ± 0.04, while those of cybrids (Cyb #1, #2a, and #2b) were 65.8 ± 4.6, 85.0 ± 7.0 and 72.0 ± 7.0 (rate constant). The citrate synthase activities of MitoCells were lost, while those of cybrid cells were maintained.

Discussion

The difficulties encountered in culturing mitochondria in vitro are due to nuclear genes being required to maintain these organelles. It is reasonable to speculate that if translated proteins from nuclear genes were provided, mitochondria might have the capacity for in vitro survival. The MitoCells which we produced lack morphologically evident nuclei but maintain mitochondrial activity and grow continuously in culture.

The anaerobic culture experiment in this study revealed that MitoCells can survive without oxygen, suggesting they produce energy through an anaerobic, rather than an aerobic, metabolic pathway. Our analyses of TCA cycle enzymes and activities revealed that the TCA cycle is not fully functional as a producer of aerobic energy in MitoCells (Fig. 2, 3).

Anaerobic mitochondria have been reported by Tielens et al[5] and Boxma et al[6]. These groups identified an anaerobic mitochondrion that produces hydrogen, possibly a missing link between mitochondria and hydrogenosomes. On the other hand, it has been hypothesized that most of the ancestral mitochondrial genome was transferred to the nucleus as the symbiotic relationship between eukaryotes and ancestral mitochondria developed. The acquisition of anaerobic metabolism is believed to be an evolutionary process[7]. Our results suggest that MitoCells may have reverted to ancestral forms to acquire an anaerobic metabolic pathway.

Considering that MitoCells were originally derived from human HeLa cells and platelets, our results suggest that human nuclear DNA may harbor an anaerobic energy production pathway independent of the aerobic pathway. Hypoxia is a clinical feature of cancer, i.e. hypoxic tumors are highly malignant, metastatic, radio- and chemoresistant and carry a poor prognosis[8]. An anaerobic metabolic pathway in hypoxic tumors has been suggested[9]. Further research analyzing this anaerobic metabolic pathway in MitoCells may elucidate the pathway operating in tumors and thereby promote the development of novel anticancer therapies.

Conclusion

MitoCells survived under anaerobic culture conditions and lost several TCA cycle-related enzymes. Only citrate synthase was present in MitoCells, but

its enzyme activity was lost. These results suggest that MitoCells produce energy employing an anaerobic metabolic pathway. Considering that MitoCells were originally derived from human cells, our results suggest that human nuclear DNA may harbor an anaerobic energy production pathway.

The authors have no conflicts of interest to declare.

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ミトコンドリア細胞の嫌気培養における生存とTCA回路の酵素に関する解析

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〔目的〕従来ミトコンドリア（Mt）は細胞外では培養不可能とされてきた．中野らはヒトの血小板とHela mitochondria-less（Rh^0）を融合させたCybrid細胞を形質変換させ，Mtの性質を保ちながら核のない細胞株の分離・増殖に成功し，ミトコンドリア細胞（MitoCell）と命名した．MitoCellの生物学的特性を見いだすため，エネルギー代謝の検討を行った．

〔対象・方法〕MitoCellの嫌気培養は嫌気培養キットでO_2濃度を0.1%以下とし，CO_2濃度が21%前後，温度は37℃で培養し光学顕微鏡にて観察した．次に，エネルギー代謝経路の検討のため，MitoCellのTCA回路のクエン酸合成酵素（CS），リンゴ酸脱水素酵素（MDH），サクシネート脱水素酵素（SDH），イソクエン酸脱水素酵素（IDH）とピルビン酸脱水素酵素（PD）をウエスタンブロット法により解析した．さらに，MitoCellの電子伝達系酵素（ETE）活性とTCA回路の酵素活性と検討するため，Cybrid細胞を対照としてETEである複合体II+III，IVとCS活性を測定した．

〔結果〕MitoCellでは嫌気的環境下でも4週間の観察で生存・増殖が認められた．ウエスタンブロット法ではMitoCellのCSのみが陽性，PD，MDH，IDH，SDHが陰性であった．酵素活性では，MitoCellは複合体II+III，IVの活性は保たれているがCS活性は欠損していた．

〔考察〕MitoCellではTCA回路が好気的代謝経路として機能していない可能性が示唆され，嫌気的代謝経路の関与が推測された．MitoCellが真核細胞であるヒト細胞から形質変換したことを考えると，ヒトの核DNAには嫌気的代謝遺伝子が保存されていると推測され，嫌気的代謝経路が賦活化されたとも考えられる．臨床上がん細胞は体内において低酸素下で存在し嫌気的代謝の関与が示唆されている．MitoCellのエネルギー代謝経路の解明は，がん細胞の治療に繋がる可能性が考えられた．

〔結論〕MitoCellでは嫌気的環境下でも生存・増殖が認められ，低酸素下で存在し嫌気的代謝の関与が示唆されているがん細胞の性質と関連している可能性が示唆された．