Original Article

Mitochondrial Activity of the Locally Established Rat Embryo Fibroblast Cell Line Through Different Passages

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Summary:

Background: Mitochondria are the cell's powerhouse, the site where the vast majority of ATP is synthesized. Mitochondrial activity represent a central checkpoint for detection of the difference between cancer cells and normal cells at the metabolic profile.

Objective: To find out if there is a correlation between ***in vitro*** transformation and mitochondrial activity, by measuring the activity during clonal evolution of the locally established rat embryo fibroblast (REF) cell line throughout studying different passages.

Materials & Methods: The (REF) cell line, was ***in vitro*** cultured. Mitochondria were isolated by differential centrifugation following Mitochondria Isolation Kit. Enzymatic activity of intact mitochondria has been measured using Cytochrome C Oxidase Activity Assay Kit. The decrease in absorbance at 550nm as horse heart ferrocytochrome c was oxidized has been monitored.

Results: Depending on the conducted colorimetric assay, cytochrome c oxidase activity was measured, for the three different passages (42, 72 and 91) of REF cell line. At 550 nm, spectral data were valued (0.247, 0.723 and 1.318) unit/ml for three passages of REF cells (42, 72 and 91) respectively.

Conclusion: There was a significant relationship between the mitochondrial activity and the age (passage number) for these ***in vitro*** cultured cells. These variations probably due to the transformational events that have been occurred during long-term continuous subculture.

Key words: Mitochondria; Cytochrome c oxidase; Rat embryo fibroblast.

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Introduction:

Although Warburg long ago had demonstrated that cancer cells are metabolically different from normal cells (1), and control steps for the altered metabolism of cancer cells have been demonstrated to lie within the glycolytic pathway (2-4), very little work has been performed to evaluate differences within the protein components of the oxidative metabolic machinery of cancer cells. Due to its central role in aerobic metabolism, cytochrome c oxidase could be expected to be altered or variably controlled in cancerous relative to normal cells (5).

The primary function of the mitochondrial electron transport chain (ETC) is the synthesis of ATP (6). Cytochrome c oxidase is the enzyme that constitutes the last step of the mitochondrial electron transport chain for the production of ATP (7). Cytochrome c (Cyt c) is a single-chain hemoprotein of 104 amino acids known for its function as a key component of electron carriers in mitochondria. (8). Apoptosis can be initiated by mitochondria, which release cytochrome c into the cytoplasm by the mediation of some cytosolic proteins as a response to extrinsic stimuli, such as oxidative insults (9,10). Cytochrome c released from apoptotic cells is an important marker of apoptosis(11).Therefore, it will be a valuable to investigate whether the metabolism of locally established rat embryo fibroblast (REF) cell line has been affected due to long-time continuous sub-culturing or not; considering cytochrome c oxidase activity as a parameter for comparison between 3 significant successive passage .

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Materials & Methods:

Cell Line Culture

The locally established normal rat embryo fibroblast (REF), established and kindly provided by Experimental therapy department, Iraqi Centre for Cancer & Medical Genetics Research (ICCMGR), was ***in vitro*** cultivated and grown according to the standard procedures of tissue culture techniques, using RPMI 1640 media (Cellgro, Mediatech, Inc., VA, USA) supplemented with 20% bovine serum (Gibco, USA) and incubated at 37˚C until the cells became confluent monolayer.

Isolation of Mitochondria: Mitochondrial isolation was carried out with a Mitochondria Isolation kit (BioChain Institute, Inc., CA, USA) all buffers and reagents were prepared using ultrapure water and all steps were carried out at 4˚C. Briefly, after the adherent cells were trypsinized, they were collected by centrifuging at 600 g for 5 min at 4˚C. Cells were washed with 10 ml ice cold PBS and centrifuged at 600 g for 5 min at 4˚C. The homogenate was transferred to 2 ml Eppendorf tube and centrifuged at 600 g for 10 min at 4˚C. The obtained supernatant was centrifuged at 12,000 g for 15 min at 4˚C. Pellet was resuspended in 50 – 100 µl mitochondria storage buffer and kept inside the refrigerator.

Measurement of Cytochrome C Oxidase Activity

The required reagents for determining the cytochrome c oxidase activity of the isolated mitochondria were conducted as described by the kit (BioChain Institute,Inc., CA, USA) . The spectrophotometer was brought at the wavelength of 550 nm before starting any reaction. The reaction started by adding 50 µl of ferrocytochrome c substrate solution and mix by inversion. Absorbencies at 550 nm were immediately recorded following a kinetic program: 5 second delay; 10 second interval; 5 readings (A_{5s}, A_{15s}, A_{25s}, A_{35s}, A_{45s}). In this assay, cytochrome c is reduced with dithiothreitol (DTT) and reoxidized by the active cytochrome c oxidase.

The maximum linear rate for both the sample and blank was calculated. The mitochondria activity was measured according to the following equation:

$$\text{Unit/ml} = \frac{\Delta A/\text{min} * \text{dilution} * 1}{\text{Vol}_{(\text{sample})} * 21.84}$$

Where:

ΔA/min= A/min(sample) – A/min(blank)

dilution = dilution factor of sample

1 = total assay volume is 1 ml

Vol(sample) = volume of sample in ml

At 550 nm reduced cytochrome c (ferrocytochrome c) has a different extinction coefficient than oxidized cytochrome c (ferricytochrome c). The difference (ΔεmM) is 21.84.

Results:

The intact mitochondria, from 3 successive passages (42, 72 and 91) of REF cell line, were isolated to measure the cytochrome c oxidase activity. By considering the blank for each passage, the absorbance readings were listed (Table 1). Depending on the collected spectral data, calculations for the linear rates during different time frame were performed for the three above mentioned passages of REF cell line (Table 2). The activity was reflected by decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. Results have showed a correlative increase between cytochrome c oxidase activity (0.247, 0.723 and 1.318) unit/ml and the successive passage number (42, 72 and 91) respectively.

Table 1: The spectral data of three different passages of REF cell line (42, 72 and 91) with respect of blank for each one, following a kinetic program: 5 second delay; 10 second interval; 5 readings.

Time point (sec)	*Absorbance at 550 nm*					
	REF (42)	*Blank*	*REF (72)*	*Blank*	*REF(91)*	*Blank*
5	*0.082*	*0.048*	*0.022*	*0.025*	*0.177*	*0.115*
15	*0.074*	*0.046*	*0.021*	*0.024*	*0.176*	*0.113*
25	*0.058*	*0.034*	*0.020*	*0.022*	*0.175*	*0.100*
35	*0.037*	*0.027*	*0.019*	*0.020*	*0.174*	*0.094*
45	*0.026*	*0.024*	*0.018*	*0.018*	*0.137*	*0.089*

Table 2: The maximum linear rate (A/min) for three different passages (42, 72 and 91) with respect of blank for each one.

Time Frame (second)	Maximum Linear Rate (A/min)					
	REF (42)	Blank	REF (72)	Blank	REF(91)	Blank
T 5s to T 15s	0.048	0.012	0.006	0.006	0.006	0.012
T 15s to T 25s	0.096	0.072	0.006	0.012	0.006	0.078
T 25s to T 35s	0.126	0.042	0.006	0.012	0.006	0.036
T 35s to T 45s	0.066	0.018	0.006	0.012	0.222	0.03

Discussion:

In mitochondria, cytochrome c plays an essential role in generation of mitochondrial transmembrane potential ($\Delta\Psi m$). This potential is essential for various functions including the production of ATP via oxidative phosphorylation (12). suggesting that mitochondria can maintain

several functions, including the generation of ATP, and may contribute to survival of the cells for prolonged periods after cytochrome c release (13). In healthy cells, ROS are kept at harmless levels by the activity of both non-enzymatic and enzymatic antioxidant systems. In the present study, there was a significant increase in the activity of cytochrome c oxidase as seen for the early passage 42 (0.247) unit/ml as compared with the older passage 72 (0.723) unit/ml. Interestingly, the REF 91 showed higher (1.318) unit/ml activity of cytochrome c oxidase activity.

Obviously, there are two different populations of cytochrome c inside the intermembrane space (IMS). Most of cytochrome c is free or weakly membrane-bound through electrostatic interactions with the negatively charged phospholipid head groups (14). Free cytochrome c or that transiently interacting with the membrane is involved in the electron transfer between membrane complexes III and IV during oxidative phosphorylation (15).

Cytochrome c oxidase functions to catalyze electron transfer from cytochrome c to molecular oxygen via four redox centers (16). Although cytochrome c oxidase only has ~20% total control over ATP synthesis (17), studies in vivo have indicated that cytochrome c oxidase represents the rate-limiting step of the electron transport chain (18). This colorimetric assay is based on observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. From the other hand, tumor cells are characterized by predominant glycolytic production of ATP, also under aerobic conditions, known as the ‘‘Warburg effect.’’ It has also been reviewed that Impaired MMP may lead to the invalidation of the apoptotic response that is found in cancer (19, 20). On theoretical grounds, this is may be due to: 1) alterations (e.g., upregulation, repression) of gene expression, resulting from genetic (e.g., amplification) or epigenetic (e.g., aberrant methylation) events; 2) loss-of function mutations; or 3) defects in the posttranslational regulation of activity resulting from intracellular localization/trafficking (e.g., inhibition of Bax translocation to mitochondria) (21). Hence, various cancer cell lines were shown to rely on glycolysis for ATP generation to different extents, but, typically, the most glycolytic tumor cells were also found to be the most aggressive ones (22). The current study may reflect transformational changes that may affect the metabolism of these cells, which may redirect our attention toward checking of normal cell lines dedicated as an in vitro model for a long time to avoid experimental misinterpretation during ***in vitro*** studies.

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