MITOCHONDRIAL DISEASE: COMPARISONS OF COX PROTEIN SUBUNIT EXPRESSION IN HUMAN FIBROBLAST CELLS

by

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Although an understanding for mitochondrial disease has developed and become more clear over the last decade, the molecular characteristics of most diseased mitochondrial cell lines are unresolved. It is with the knowledge of COX subunit expression patterns in healthy (MRC5) cells and in chemically derived, diseased (MRC5-Rho0) cells that molecular based diagnostic tools will be available for clinical use. Research that I collaborated on in the Monoclonal Antibody Facility (MAF) shows evidence of human fibroblast cell line 5065 having mitochondrial DNA (mDNA) depletion and thus, having mitochondrial disease. The patient cell line was cultured in vitro, and its expression pattern of COX protein subunits was compared to both that of the MRC5 and MRC5-Rho0 cell lines.

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CHAPTER ONE

Introduction

Studies of human mitochondrial DNA were stimulated by the discovery of mitochondrial based diseases and their connection to mutations in mDNA. It was in 1988 that these mutations in the mitochondrial DNA genome were first discovered. Research conducted in the past decade has led to a better understanding of the small cellular organelle which hosts its own DNA. The goal of my research was to assist in characterizing the necessary protein components of the mitochondrion, or lack there of, that can be used as diagnostic tools for recognizing mitochondrial disease. I contributed to research conducted by Dr. Mike Marusich and his colleagues, in particular, Dr. Rodrick Capaldi and members of the Monoclonal Antibody Facility at the Institute of Molecular Biology. This research, and previous work, shows that mitochondrial cytochrome c oxidase, COX, degenerates in human fibroblast patient lines with cell proliferation in vitro; while COX of normal human fibroblast cells does not.

The mitochondrion is an organelle of eukaryotic cells. Mitochondria are approximately one micrometer in diameter and one to ten micrometers in length. They are usually visualized in the electron microscope as oval shaped organelles that can easily change shape by stretching out or shrinking up lengthwise. Sometimes mitochondria of particular cell types are certain shapes, more rounded or more lengthened.

The origins of the mitochondria are unlike that of most cell organelles. Due to their unique ability to divide and replicate their DNA and transcribe and translate their own RNA it is thought that the ancestral form of mitochondria existed as independent organisms. Also, structural similarities of mitochondrial DNA to prokaryotic DNA support this theory. At some point these progenitor cells were incorporated or engulfed by larger cells. Mitochondria are still considered to be semi-autonomous organelles because of their ability to grow and divide (Johns et al, 1995). Although this may be true, mitochondria are no longer self-sufficient and depend on the cell to carry out many of their important functions.

The mitochondrion is enclosed by a double membrane, the inner and outer membranes separating the intermembrane compartment

and matrix. The outer membrane separates the organelle from the surrounding cell cytoplasm and selects for transportation of only particular substances across its lipid bilayer. The importance of these membranes is to provide separate compartments that can contain very different substances. The inner membrane is folded many times into what are called cristae. The folds of cristae are necessary for providing a large surface area inside the cell. The surface area inside the cell is important for the manufacturing of huge quantities of chemical energy that fuels the body. These folds are easily visible in the following figure:

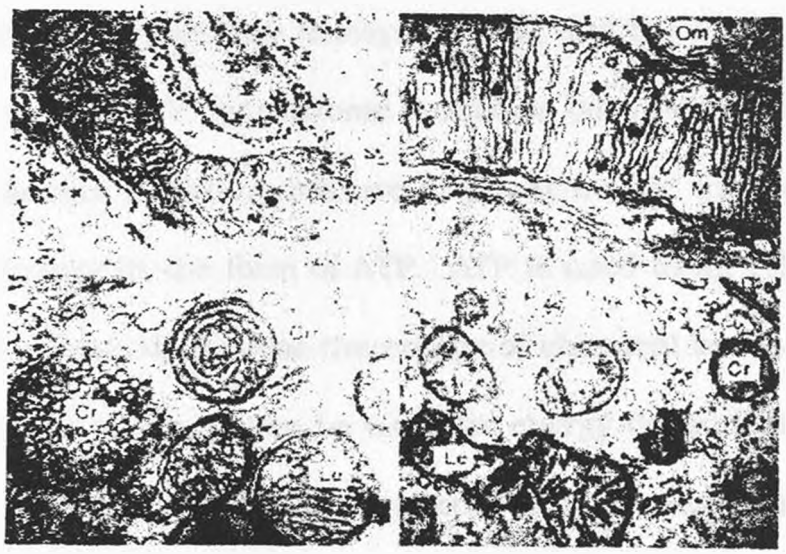


Figure 1. Folded Cristae of Mitochondria (Internet 2).

The space surrounded by the inner membrane is labeled the matrix , it contains mDNA and mitochondrial ribosomes.

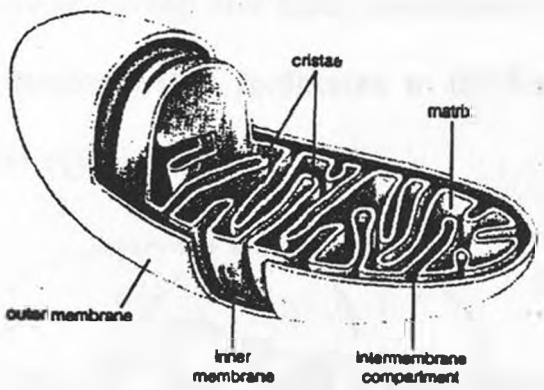


Figure 2. A Mitochondrion (Internet 1).

Within the mitochondrial inner membrane lie the molecular components of the electron transport chain and the focus of my research, complex IV, cytochrome c oxidase (also referred to as COX).

In essence mitochondria are a "power house" for producing chemical energy in the form of ATP. ATP is used in all cells of the body. Mitochondria harness the energy of chemical bonds and allow the release of this energy to be used for energy dependent cellular activities. To better understand mitochondrial disease it is necessary to be familiar with mitochondrial functions. Through oxidative phosphorylation, a process performed by the electron transport chain,

healthy and non-mutated mitochondria utilize energy obtained from food. The energy contained in food chemical bonds is released and used to pump protons across the mitochondrion's inner membrane, creating a proton gradient that facilitates in the formation of ATP (Wallace 1992), see figure 3:

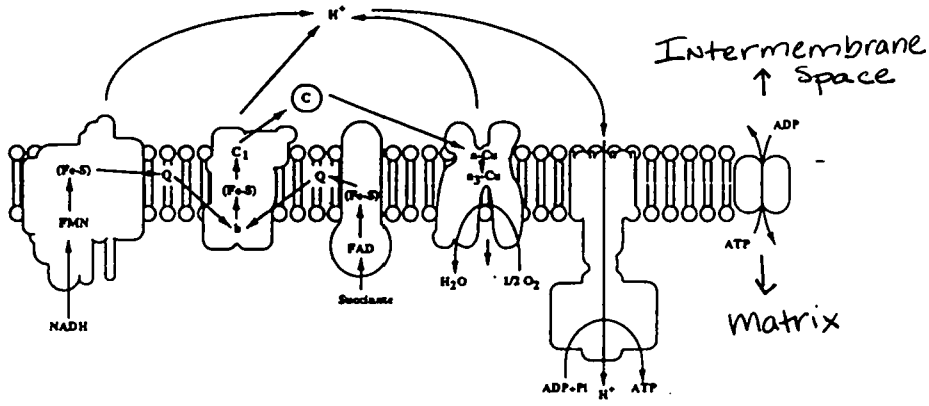


Figure 3. The Electron Transport Chain (Wallace 1992).

ATP is then transferred from the cell to the areas of the body requiring ready energy. The main organ tissues requiring high levels of ATP are: skeletal muscle, the brain, the heart, eye tissues, liver, kidney, pancreas, blood, inner ear, and colon (Johns et al, 1995). The vast number of tissues that rely on ready ATP make it is easy to understand the profound effects caused by mutated mitochondria that cannot produce a threshold amount of ATP. Figure 4 illustrates the

various tissues that ATP production effects:

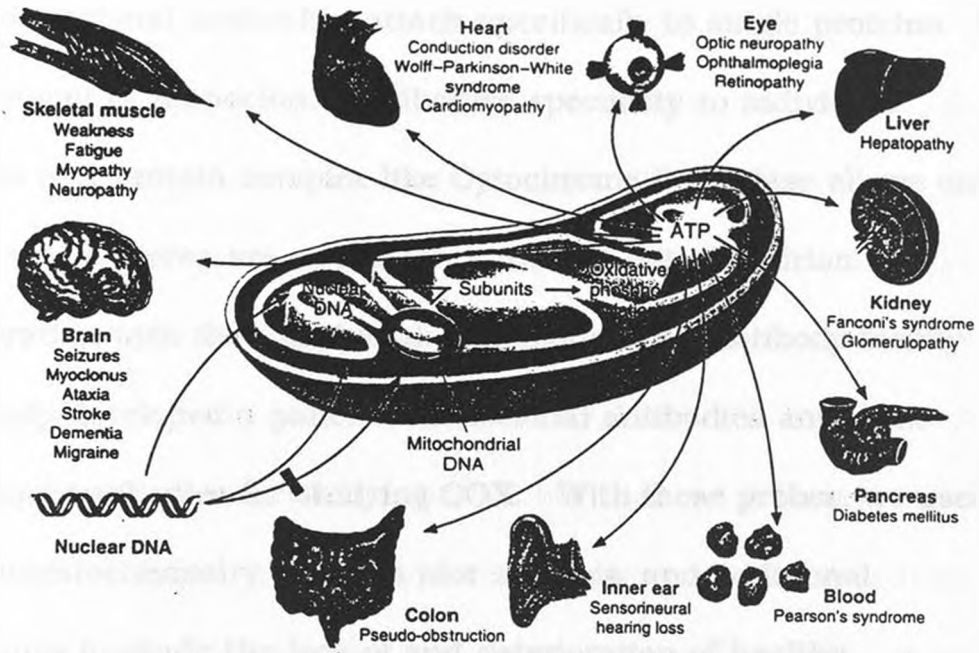


Figure 4. Tissues that Rely on ATP Production (Johns et al, 1995).

Cytochrome c oxidase is the fourth complex of the electron transport chain. COX is composed of 13 different protein subunits. Three of these subunits, I, II, and III, are encoded by mDNA. The other ten are encoded by nuclear DNA (Capaldi et al, 1995). In the study of mitochondrial disease it has been found that defective COX function is implicated in the malfunction of the electron transport chain which leads to nonviable mitochondria. These mitochondria are not producing the threshold amount of ATP needed for a cell to

properly function.

Monoclonal antibodies attach specifically to single proteins. The development of monoclonal antibodies specificity to individual proteins of a protein complex like Cytochrome C Oxidase allows one to assess which pieces are missing in a mutant mitochondrion. In collaboration with the Capaldi lab, the monoclonal antibody facility previously developed a panel of monoclonal antibodies and some polyclonal antibodies for studying COX. With these probes, we used immunohistochemistry, western blot analysis, and additional techniques to study the lack of and deterioration of healthy mitochondria in patient cell lines. Immunohistochemical assays allow for labeling of specific mitochondrial proteins in the fibroblast cells with monoclonal antibodies. Secondary antibodies, labeled with fluorescence, attach to the monoclonal antibodies. Using a specialized microscope, the Axioplan, the mitochondria labeled with fluorescence from the secondary antibody can be seen.

Immunohistochemistry (IHC) allows us to visualize whether particular protein subunits are present and to see exactly where they are in the cell. IHC is the technique of fixation of labeled cells onto glass slides which can later be viewed under the Axioplan microscope.

This technique has been formulated and personalized for our purposes. It involves the following steps: rinsing the cell cultures, fixing them with paraformaldehyde, blocking them with the primary monoclonal antibodies, then blocking them with the fluorescence labeled secondary antibodies, and finally mounting them to be viewed under the microscope. Data can be gathered from using this technique to compare a cell's mitochondrial molecular state at different levels of cell proliferation, mainly "young" (few cell divisions) versus "old" (many cell divisions). This is precisely what has been done with cell line 5065 and it has allowed us to better characterize COX in the patient line throughout cell proliferation as compared with healthy, non-patient cells.

Although it has long been known that mitochondria contain their own DNA, it was not clearly understood that mDNA could harbor profound genetic defects that lead to disease. These defects were first discovered in 1988 and they produce a variety of diseases (Wallace 1997). In the years since then many new diseases have been diagnosed and linked to mDNA. These disorders and abnormalities can affect every organ and tissue of the body, are very difficult to predict, and even more difficult to diagnose.

Unlike DNA of the cell nucleus which is divided with precise equality to each daughter cell, mitochondrial DNA is divided at random. An individual cell contains hundreds to thousands of mitochondria, each with 5 to 10 copies of mitochondrial DNA. Each mDNA may be a wild type or mutant. A mitochondrion with both wild type and mutant mDNAs mixed is heteroplasmic versus homoplasmic. Mitochondria are found throughout the cytoplasm of most cells and at cell division the mitochondria are shuffled without organization to one daughter cell or the other. It is in this chance division of mitochondria to the two daughter cells that bad mitochondria will sometimes accumulate in a particular tissue, or may be spread evenly through the body.

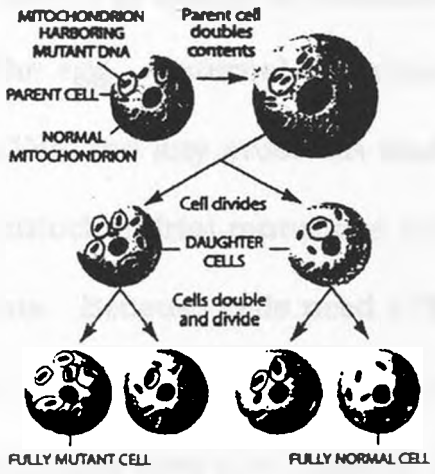


Figure 5. Cell and Mitochondrial Division (Wallace 1997).

Figure 5 demonstrates the way that some cells can eventually contain fully mutant mitochondria. When these cells develop they many become concentrated into a particular tissue or organ. The tissue concentrated with mutant mitochondria would be unable function at a full capacity.

For some time it has been known that mitochondrial related diseases are maternally inherited. Research studies have shown that mitochondria and thus, mitochondrial DNA are strictly maternally inherited (Giles et al, 1980 and Hauswirth and Laipis, 1985). This suggests that mitochondria passed to the fetus are donated from the cytoplasm of the maternal egg. The sperm, which donates an equal amount of nuclear DNA to the female egg has little cytoplasm. It is possible that the sperm is unable to contribute cytoplasm or mitochondria to the egg. Maternal inheritance is therefore primarily responsible for mDNA and any problems that may exist. It is very likely that many mitochondrial mutations go unnoticed with early abortion of the fetus. Because cells need ATP to carry out many general functions, if the maternal egg starts out with too many "bad" or mutated mitochondria then it is unlikely that the fetus will develop. If the maternal egg begins with only a few "bad" mitochondria, then

these mitochondria could be passed along from cell division to cell division, sometimes dispersed throughout the body and sometimes concentrated to particular areas. This knowledge of maternal inheritance and cellular division of mitochondria provide useful tools for diagnosis of mitochondrial disease.

CHAPTER 2

Materials and Methods

The MAF uses a variety of cell types to conduct experiments that provide a model for the molecular makeup of mitochondrial proteins and a basis for diagnosing mitochondrial disease. The patient cell line 5065 was obtained through a tissue biopsy that generated fibroblast cells from the patient. The actual number of population doublings (PD) is not known, therefore, these cells are regarded as being at a state of 0 PDs upon receipt by the MAF. Population doublings are an indication of how many times the cells have divided and thus can be used as indication of their age in vitro.

MRC5 fibroblasts are cells that contain healthy viable mitochondria with no genetic defects. These cells were obtained from the American Type Culture Collection and upon receipt were at an age of 25 PDs (Marusich et al, 1997). For studies in the MAF the MRC5 fibroblasts were used at PD level no greater than 45. These healthy control cells maintain stable growth rates and respiratory chain protein expression patterns in this PD range.

MRC5-Rho0 fibroblasts were derived from MRC5 fibroblasts through a selective culture using ethidium bromide at 50 ng/ml over a period of 12-22 PD (see methods of Marusich et al, 1997). The result of subculturing the MRC5 cells in ethidium bromide were MRC5-Rho0 fibroblasts which do not contain mitochondrial DNA and therefore lack important components of the respiratory chain protein complex. These fibroblasts are used as controls in comparisons with patient cell lines.

All cell types are grown in a standard medium for fibroblast cell culture 10F-HGDMEM-SU, High Glucose Dulbecco's Modified Eagle Medium, supplemented with 10mM HEPES, 50μM 2-mercaptoethanol (3.5 μl/L), 20μM ethanolamine (1.2μl/L), 1μg/ml bovine insulin, 10% fetal calf serum, and 50μg/ml uridine.

Cells are grown on sterile culture dishes in the above specified medium until fully confluent. Before cells begin to overlap they should be subcultured at a 1-2 or 1-4 spilt onto new sterile culture dishes. The culture medium is removed by rinsing twice with sterile CMF-PBS. Then a trypsin solution is added to the culture dish to help the cells unfasten. After 5-10 minutes of incubating at 37°C most of the cells would be free of the dish and can be removed. An equal volume

of culture medium containing 10% fetal calf serum is added to the dish. With a pasture pipette the solutions are used to rinse the dish and remove the cells. After removing the cells to a sterile centrifuge tube they are spun down and then the supernatant is discarded to remove all of the trypsin. The cell pellet is resuspended in fresh culture medium and distributed to two or four sterile culture dishes.

One population doubling is achieved when a 1-2 split of the cells into two new culture dishes becomes confluent. The cells adhere to the dish quickly and become confluent twice as fast as when doing a 1-4 split. Aging the cells only one population doubling is useful when you need healthy confluent cells quickly, but it does require more work and twice as much time. For most of the experiments a 1-4 split is used and the cells age two PDs between each subculture.

When subculturing cells not only is the population doubling increased, but also the volume of cells. In a matter of a few days confluent cells in one dish generate confluent cells in four dishes. For this reason usually one dish is subcultured to increase the level of PDs and the other three dishes are frozen for stock supplies, made into a cell extract, or used for immunohistochemical assays.

When freezing the living fibroblast cells for stock supplies it is

important to follow the procedure exactly to insure that the cells are still living when thawed. The cells are removed from the culture dish as described for the subculture. After centrifuging the cell suspension the supernatant is discarded and the cell pellet is resuspended into a desired volume of freezing medium. The desired volume of freezing medium is calculated by counting the cell density, recording the total cell number and the yield of cells per dish. The cells should be distributed to four or six NUNC cryotubes containing 1 ml of freezing medium each. The freezing medium consists of fresh, sterile 20F-HGDMEM-SU containing 10% DMSO at 37°C. The NUNC cryotubes are placed in a freezing container, labeled, and then placed in the -85°C freezer. After several days in the freezing container they will be removed and placed in a designated location in the -85°C freezer.

To thaw the frozen fibroblast cells fresh 10F HGDMEM-SU culture medium is placed into a sterile centrifuge tube. The frozen vial of desired cells is removed from the -85°C freezer and quickly thawed by immersing in the 37°C water bath. Once the vial has thawed the contents are added to the culture medium in the centrifuge tube. A pipette is used to rinse the vial containing the cells. This is to be sure that all of the cells have been collected. To remove

the freezing medium from the culture medium containing the cells the suspension is centrifuged and the supernatant is discarded. The cell pellet is then resuspended in a desired volume of fresh culture medium. This volume is calculated based on the cell count which is recorded on each NUNC cryotube. Usually, but not always, the vial contains 20% of the cells from a 150mm culture dish and therefore will be plated at a 1-5 split. The 1-5 split must be considered when calculating the corresponding population doubling. As with cell subcultures the new culture dish containing thawed cells must be kept at 37°C in a CO2 incubator. The culture dish should be checked every day to watch for and determine when the cells will reach confluency. Every couple of days the culture medium should be changed to fresh culture medium to insure that the cells stay healthy and viable.

Cell extracts are prepared from confluent culture dishes and are later used for western blot analysis. Again the confluent cells are trypsinized and removed from the culture dish. After centrifuging and discarding the supernatant the cell pellet should be resuspended in fresh culture medium and a cell count should be taken to calculate the total cell number. Once again the cell suspension is spun down and the supernatant discarded. This time the cell pellet should be

resuspended in sterile CMF-PBS. This cell suspension is then centrifuged and the supernatant discarded. This step is to rinse the culture medium from the cells. The rinsed cell pellet is suspended in a solution of CMF-PBS containing protease inhibitors (PBS-I), leupeptin, pepstatin, and PMSF at 4°C. This cell suspension is transferred to a Sarstadt microfuge tube and centrifuged at 14,000 rpm for a minute. The supernatant is discarded and the cells are resuspended in a fresh solution of PBS-I plus sodium lauryl maltoside (PBS-ISLM), a detergent. The cell suspension containing detergent is incubated for 30 minutes at 4°C on a rotary mixer. The PBS-ISLM causes the cell membranes to break down and release the cell's cytoplasm along with mitochondria and other cytoplasmic proteins. To separate the cell extract from the cell fragments, nuclei, and other "heavy" proteins the cell suspension is centrifuged at 14,000 rpm for 20 minutes. After this final spin down the supernatant (containing mitochondria) is saved, labeled, and frozen in the -85°C freezer for future western blot analysis.

While western blot analysis shows COX subunit expression for the cell population average, immunohistochemistry is conducted to show differences or changes in COX protein subunit expression at

various levels of cell proliferation for single cells. Subcultured cells from confluent dishes are distributed onto slides at a 1-4 split. The cells are placed in wells of agar that are sterilized and secured onto acid cleaned, glass slides. The cells adhere to the slide and begin to grow and divide in the well. When the cells reach 50% confluency they are ready to be used for immunohistochemical assays.

Two slides with 50% confluent cells are treated with Mito Tracker to insure the presence of mitochondria. Mito Tracker is a mitochondrion-selective fluorescent dye that must be applied to living cells. The slide's well medium is replaced with fresh medium containing diluted Mito Tracker and is incubated at 37°C for 45 minutes in the CO2 incubator. To be sure that excess Mito Tracker is rinsed off after incubation the slides are rinsed three times, ten minutes each, with fresh medium warmed to 37°C. The two slides treated with Mito Tracker can now proceed through the immunohistochemical assay.

Along with the slides treated with Mito Tracker a variety of other slides with various cell types will be probed with antibodies. It is important to plan out the immunohistochemical experiment early and decide exactly how many slides will be needed to test all of the

antibodies and controls. The number of slides that are needed should be doubled and each experiment run twice to leave little room for error. The slides are first rinsed with warm Hanks-Hepes, at 37°C, before being fixed at 4°C with fresh 4% paraformaldehyde in Hanks Hepes for 20 minutes. Following this preliminary fix I replace the solution with 4% paraformaldehyde in 0.1 M Na-Borate at pH 11. The slides incubate for sixty minutes in the final fix at 4°C. These fixations allow the cells to adhere to the slides and maintain the cell's organelles and structural characteristics.

After fixing the cells to the slides they are washed several times with CMF-PBS to rinse away the fix solution. Next, the slides are permeabilized for 15 minutes in Acetone at -20°C. Again they are washed with 4 changes of CMF-PBS and are prepared for treatment with the monoclonal antibodies. The slides are then blocked for one hour in 10% Normal Goat Serum (NGS) in CMF-PBS. Following the NGS block the monoclonal antibody (MAb) solutions are added to the wells and the slides are incubated overnight in a humid chamber at 4°C. The unbound MAbs are washed off with 4 changes of CMF-PBS and then the slides are blocked for 5 minutes in 10% NGS. Next, the Goat Anti Mouse (GAM) solution with Hoechst dye 33258 is applied

and the slides are incubated for 2 hours at room temperature (RT). The Goat Anti Mouse solution is a secondary antibody that binds to the monoclonal antibodies and the Hoechst dye stains all of the cell nuclei. This allows the nuclei of cells with "bad" mitochondria to be visible even when the mitochondria are not.

Finally, the secondary antibody solution is rinsed away, the slides are mounted with Fluormount 90, and are stored in the -20°C freezer until they can be viewed under the Axioplan microscope. It is important to keep the slides out of the light because it degrades their fluorescent properties.

CHAPTER 3

MAF Research and Discussion

Mitochondrial disease is very difficult to diagnose due to the wide variety of tissues that a lack of ATP effects (Johns et al, 1995). Collaboration at other institutions has provided the Monoclonal Antibody Facility (MAF) with about two dozen human fibroblast cell samples derived from patients with known or suspected heritable mitochondrial diseases. Tissue biopsies of living cells from the patients are stored as frozen stocks and are available for our research and study.

The particular cell line 5065 was initially reported to have COX cardiac deficiency (Marusich 1995). Two other cell lines, MRC5 and MRC5-Rho0 are used in our research as controls. The MRC5 cells contain completely "normal" mitochondria, having all of the necessary proteins to function, and produce a threshold amount of ATP. In studies that I conducted it was again shown that the wild type MRC5 cells will go through 50 to 60 population doublings before they slow down, begin producing cell debris, and die out. This is a well known

characteristic of normal human fibroblasts that is sometimes called aging in vitro. In contrast, for example, cancer cells never stop dividing.

The MRC5-Rho0 cell line has been developed in the MAF and is derived from normal MRC5 cells; through chemical treatment MRC5-Rho0 is void of all mitochondrial DNA (Marusich 1995). It does not produce a threshold amount of ATP and needs to be supplemented with a special culture medium to be viable. The MRC5-Rho0 cells are true examples of mitochondrial diseased fibroblasts and can be used in comparison with suspected mitochondrial diseased patient cell lines. If a suspected patient line shows similar COX protein expression to that of MRC5-Rho0, then this tool can be diagnostic in determining that the patient is afflicted by a mitochondrial disease versus some other ailment. Our ongoing goal is to gain a better understanding of the particular molecular deficiencies of COX in MRC5-Rho0 cells and to further characterize mitochondrial disease.

For immunohistochemical purposes we have four monoclonal antibodies that bind the I, II, IV, and VIc protein subunits of COX. We would prefer to have antibodies for each of the thirteen subunits, but due to possible conformational changes of the epitopes in the

immunohistochemical fixation process these antibodies are not yet available (Marusich 1995). For western blot analysis all 13 of the monoclonal antibodies are available. These are used to give us a general idea of the particular protein's average expression pattern for cell populations. The antibodies that we do use for immunohistochemistry are sufficient for the reason that two of them, anti-I and anti-II, bind mitochondrial DNA encoded proteins and the other two, anti-IV and anti-VIc, bind nuclear DNA encoded proteins. These antibodies are are representative examples of two different classes of proteins. This difference allows us to compare changes in proteins of the mitochondrion that are encoded by the nuclear DNA versus changes in those encoded by mitochondrial DNA.

Although we do have two representative classes of proteins that interact with the monoclonal antibodies, it would be helpful to have a monoclonal antibody for each protein subunit of the COX protein complex. One thing that I worked on in my research with the MAF was to find ways to make the monoclonal antibodies that could be detected with western blot analysis work for immunohistochemistry.

It is thought that the fixation process described in the materials and methods section might distort some of the protein subunits of

COX and cause them to be unrecognizable by their monoclonal antibody even though they are actually present. With this in mind I looked into additional ways of fixing the cells that worked for other labs using monoclonal antibodies.

Normally the fixation process is done at 4°C, this seems to work best for the antibodies that we already use, but in some cases extreme heat during fixation allows other monoclonal antibodies to be detected (Shi et al, 1996). This was true in the case of monoclonal antibody Va. After several trials I was able to maintain adhesion of the cells to the slides throughout the heated fixation process and to successfully bind monoclonal antibody Va to the protein. It is likely that by attempting different fixation and sub-fixation techniques that additional monoclonal antibodies will become available for the purpose of immunohistochemistry.

As I mentioned previously the MRC5-Rho0 cell line provides a characteristic pattern for COX subunit expression in mDNA depleted cells, those that are considered to have mitochondrial disease. This is also the case for the MRC5 cell line, which provides the characteristic pattern of COX subunit expression in normal, non-diseased cells. These two control cell lines provide confirmation of the structural

make-up of the COX protein complex and also support the use of the subunit expression pattern as a diagnostic tool.

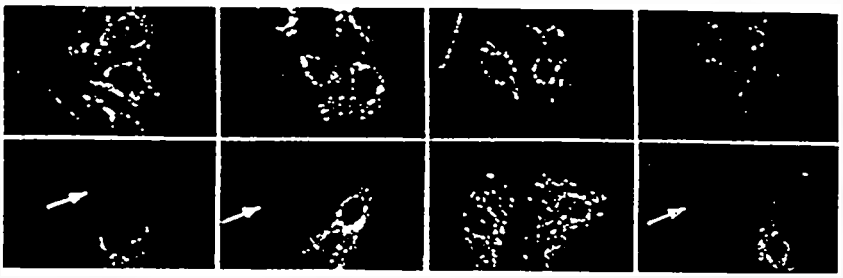
The two control cell lines, MRC5 and MRC5-Rho0, helped to characterize the defect in patient cell line 5065. In cell line 5065 we saw a quantitative reduction in cells expressing COX protein subunits with increasing population doublings. This observation suggested a mitochondrial DNA depletion, and thus mitochondrial disease. With DNA tests called southern blot analysis the hypothesis of mitochondrial DNA depletion was confirmed. It was in this way that the characteristic COX expression pattern was used to diagnose patient cell line 5065 as a mitochondrial disease.

When the MAF receives cell tissue samples, such as 5065, we designate them to be 0 population doublings although, they have actually gone through an undeterminable number of PDs as the embryo developed into a fetus. We freeze some of the tissue samples at this young age and continue to proliferate the others. With immunohistochemical technique patient cell line 5065 shows an approximate loss of 50% of properly functioning mitochondria at an initially low population doubling. Over a rather short period of time and few population doublings cell line 5065 loses nearly 100% of its

properly functioning mitochondria. It is at this point that the patient cell line is very similar, if not identical, to the control line MRC5-Rho0. Both lines now lack mitochondrial DNA, but one was derived through culture of diseased cells and one through chemical means. From these observations it is inferred that mitochondrial depletion can have a late onset, occurring after the birth of the fetus, and can also be very rapid, happening after only a few population doublings in vitro and a few years in vivo.

The loss of viable mitochondria in patient cell line 5065 can be detected in figure 6 (following page) when compared to normal MRC5 cells. The mitochondria are visualized here as bright fibril type structures using the Texas-red filter on the Axioplan. They create a meshwork surrounding the nuclei. The nuclei appear as dark spots, because their fluorescent label cannot be seen with the Texas-Red filter. The 5065 patient cell line at a low level of PDs appears to have a heterozygous mixture of approximately 50% of cells with healthy mitochondria and 50% of cells with unhealthy mitochondria, for protein subunits I, II, and VIc. The arrows in the 5065, patient cell line, figures are pointing at nuclei of cells with unlabeled mitochondria. In comparison, the MRC5 cell line indicates

mitochondrial protein staining in 100% of the shown cells.



All normal MRC-5 cells express subunits I, II, IV and VIc. In contrast, cell line #5065 is a heterogeneous population: some cells express all subunits, while other cells express only subunit IV. Arrows indicate negative cells.

Figure 6. mDNA Depletion of Cell Line 5065 (Marusich 1995).

Over time and population doublings the number of cells with healthy mitochondria drastically decreases in patient cell line 5065, while MRC5 cell's mitochondria stay healthy. This provides quantitative evidence for COX protein subunit deterioration with proliferation of the patient cell line and begins to give a molecular basis for this particular mitochondrial disease.

With more research into the molecular characterization of different mitochondrial diseases will come tools for diagnosis. It has already been shown here that by comparing a patient cell line of suspected mitochondrial diseased tissue to the MRC5-Rho0 cell line

we can determine whether the affliction is caused by mitochondrial depletion. This procedure helps to rule-out other diseases which could show the same phenotype as a mitochondrial disease. I hope to see this research, and the comprehensive characterization of COX in relation to mitochondrial disease, used to create tools for early diagnosis and eventually cures for mitochondrial diseases.

APPENDIX A

Glossary of Terms

Antibody - any of numerous protein molecules produced by B cells as a primary immune defense, each kind having a uniquely shaped site that combines with a foreign antigen.

ATP - adenosine triphosphate: a nucleotide that is the primary source of energy in all living cells because of its function in donating a phosphate group during biochemical activities; composed of adenosine, ribose and three phosphate groups.

Complementation - the compensation of one wild-type, or good, gene for a gene with that mutation.

COX, cytochrome c oxidase - the third protein subunit of the electron transport chain, contains three mitochondrial encoded subunits and 10 nuclear encoded subunits.

Electron transport chain - mechanism functioning within the inner-membrane of the mitochondria to produce ATP energy.

Fibroblast - a cell that contributes to the formation of the connective tissue fibers.

Fluorescence - the light, radiation emitting property of the secondary

antibodies, which can be detected by a specially filtered microscope.

Genome - a full hapliod set of chromosomes with all of its genes; the total genetic constitution of a cell or organism.

mDNA - (mitochondrial) deoxyribonucleic acid: an extremely long, double-stranded nucleic acid molecule arranged as a double helix that is the main constituent of the chromosome and that carries the genes as segments along its strands.

Mitochondria - an organelle in the cell cytoplasm that has its own DNA and that produces enzymes essential for energy metabolism.

Monoclonal antibodies - antibodies which recognize and bind to one specific protein subunit, give us the ability to detect whether particular protein are present.

mRNA - (mitochondrial) ribonucleic acid: any of a class of single-stranded nucleic acid molecules composed of ribose and uracil, important in protein synthesis and in the transmission of genetic information transcribed from DNA.

Mutation - a sudden departure from the parent type in one or more heritable characteristics, caused by a change in a gene or

chromosome.

Organelle - "small organ", specialized structures preforming specialized functions found in eukaryotic (many cell organism) cells.

Oxidative phosphorylation - the aerobic synthesis, coupled to electron transport, of ATP from phosphate and ADP.

Polyclonal antibodies - antibodies which recognize and bind to specific protein groups.

Protein - composed of twenty or more amino acids linked in one or more long chains, the final shape is determined by the side chains of the amino acids and their chemical attachments.

Proton - a positively charged elementary particle found in all atomic nuclei, having a positive charge and also an important part of the electron transport chain.

Rho-cell line - viable human fibroblast cells containing a nucleus and other functioning organelles, but having no functional mitochondrial DNA, kept viable via supplemented medium.

Succinate dehydrogenase - complex II of the electron transport chain, composed of four protein subunits.

BIBLIOGRAPHY

Capaldi RA (1988) Mitochondrial myopathies and respiratory chain proteins. Trends Biochem Sci 13:144-147.

Capaldi RA (1991) Structural features of the mitochondrial electron transfer chain. Curr Opin Struct Biol 1:562-568.

Capaldi RA, MF Marusich, and J-W Taanman (1995) Mammalian Cytochrome-c Oxidase: characterization of enzyme and immunological detection of subunits in tissue extracts and whole cells. Methods In Enzymology 260:117-132.

DiMauro S, and CT Moraes (1993) Mitochondrial encephalmyopathies. Arch Neurol 50:1197-1208.

Giles RE, H Blanc, HM Cann, and DC Wallace (1980) Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci 77:6715-6719.

Hauswirth WW, and PJ Laipis (1985) Transmission genetics of mammalian mitochondria: a molecular model and experimental evidence. Elsevier Science B. V. 2:49-59.

Johns DR (1996) The other human genome: mitochondrial DNA and disease. Nature Med 2:1065-1068.

Johns DR, JS Flier, and LH Underhill (1995) Mitochondrial DNA and disease. New Engl Jorn Med Sept 7:683-644.

Luft R (1994) The development of mitochondrial medicine. Proc Natl Acad Sci 91:8731-8738.

Marusich MF, BH Robinson, J-W Taanman, SJ Kim, R Schillace, JL Smith, and RA Capaldi (1997) Expression of mtDNA and nDNA encoded respiratory chain proteins in chemically and genetically-derived Rho0 human fibroblasts. Biochimica et Biophysica Acta 61677.

Marusich MF (1995) Human mitochondrial diseases: immunochemical diagnosis and analysis. Oregon Medical Research Foundation.

Shi S-R, RJ Cote, L Young, SA Iman, and CL Taylor (1996) Use of pH 9.5 tris HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. Biotecnic and Histochemistry 71:190-195.

Taanman J-W, MD Burton, MF Marusich, NG Kennaway, and RA Capaldi (1996) Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome c oxidase subunits in chronic progressive external ophthalmoplegia. Biochem Biophys Acta 1315:199-207.

Taanman J-W, RE Hall, C Tang, MF Marusich, NG Kennaway, and RA Capaldi (1993) Tissue distribution of cytochrome c oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. Biochem Biophys Acta 1225:95-100.

Wallace DC (1992) Diseases of the mitochondrial DNA. Annu Rev Biochem 61:1175-1212.

Wallace DC (1994) Mitochondrial DNA sequence variation in human evolution and disease. Proc Natl Acad Sci 91:8739-8746.

Wallace DC (1997) Mitochondrial DNA in aging and disease. Scientific Amer Aug: 40-47.

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1 http://falk.ucdavis.edu/bio10/dia_mito.gif

2 http://www.usc.edu/hsc/med-sch/images/mic