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Long-Term Analysis of Differentiation in Human Myoblasts Repopulated with Mitochondria Harboring mtDNA Mutations

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Short-term analysis of myogenesis in respiration-deficient myoblasts demonstrated that respiratory chain dysfunction impairs muscle differentiation. To investigate long-term consequences of a deficiency in oxidative phosphorylation on myogenesis, we quantitated myoblast fusion and expression of sarcomeric myosin in respiration-deficient myogenic cybrids. We produced viable myoblasts harboring exclusively mtDNA with large-scale deletions by treating wild-type myoblasts with rhodamine 6G and fusing them with cytoplasts homoplasmic for two different mutated mtDNAs. Recovery of growth in transmitochondrial myoblasts demonstrated that respiratory chain function is not required for recovery of rhodamine 6G-treated cells. Both transmitochondrial respiration-deficient cultures exhibited impaired myoblast fusion. Expression of sarcomeric myosin was also delayed in deficient myoblasts. However, 4 weeks after induction of differentiation, one cell line was able to quantitatively recover its capacity to form postmitotic muscle cells. This indicates that while oxidative phosphorylation is an important source of ATP for muscle development, myoblast differentiation can be supported entirely by glycolysis.

Mitochondria are cellular organelles with many metabolic functions, including oxidative phosphorylation, an important source of ATP production. While the nuclear genome is responsible for the control of mitochondrial biogenesis, the mtDNA contributes towards the differentiation of this organelle for its role in oxidative phosphorylation. The mtDNA encodes only part of the machinery necessary for the expression of the 13 mtDNA-encoded subunits of the respiratory chain enzymes, namely the large and small rRNAs and 22 tRNAs. The remaining proteins responsible for mtDNA replication and transcription and mitochondrial translation are nuclear DNA encoded (1).

During skeletal muscle development, mtDNA content as well as the level of expression of mitochondrial proteins increase, suggesting that ATP generated by respiratory chain activity is an important source of energy for muscle development. Myogenesis is not an exclusive phenomenon of early development but occurs throughout life. Both muscle growth and regeneration are accomplished by proliferation of satellite cells and their subsequent fusion to form new myofibers by a developmental program similar to that of embryonic muscle cells. Therefore, myogenic cells in culture constitute a model system to study muscle differentiation.

Boulet *et al.* (2) observed myotube formation in respiratory chain-deficient myoblast cultures derived from MERRF (myoclonic epilepsy and ragged red fibers) patients. However, no quantitative analyses of myogenic differentiation were conducted. Herzberg *et al.* (3) performed short-term ethidium bromide treatment of cultured myoblasts to investigate the effects of respiratory chain deficiency on muscle differentiation. They found that respiration-deficient myoblasts showed impaired fusion capacity and decreased levels of muscle-specific creatine kinase after 6 days of induction of differentiation. Although the interruption of mtDNA replication is a well known effect of ethidium bromide, the possibility of additional effects of the drug contributing to the observed impairment of myogenesis

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could not be eliminated. Furthermore, long-term studies of muscle differentiation were not performed.

The mitochondrial encephalomyopathies are a heterogeneous group of diseases caused by nuclear DNA mutations and mtDNA mutations (4). The study of myogenic differentiation in respiration-deficient human muscle satellite cells harboring pathogenic mtDNA mutations would permit the role of respiratory chain derived energy production in muscle cell differentiation to be analyzed. In addition, studying the development and biological behavior of respiration-deficient myoblasts may reveal mechanisms by which myogenic cells overcome metabolic deficiencies and adapt to different metabolic environments.

Long-term ethidium bromide treatment has been used to produce myogenic cells partially depleted of mtDNA (3). While this culture system is useful to examine the effects of mtDNA depletion and respiratory chain deficiency on cultured muscle, we find that this model has limited utility for producing transmitochondrial myogenic cells. It is difficult to achieve complete elimination of the autologous mtDNA with ethidium bromide treatment and the residual levels of mtDNA gradually increase over time following withdrawal of ethidium bromide (unpublished observations). Furthermore, ethidium bromide requires continuous cell proliferation to eliminate mtDNA, which is difficult to achieve in muscle cultures, where proliferating and post-mitotic cells co-exist.

A method that could quickly eliminate the mtDNA population of cells is therefore required to overcome the limitations of the method of depleting cells of mtDNA with ethidium bromide. Ziegler and Davidson (5) reported that pretreatment of cells with rhodamine 6G limited the cytoplasmic contribution of the treated cells in hybrid and cybrid crosses. Although the drug was lethally toxic, without any apparent effect on the nuclear DNA, treated cells could be rescued by fusion with untreated whole cells or enucleated cells (cytoplasts). Subsequent molecular genetic studies have shown that rhodamine 6G treatment can eliminate the contribution of parental cells' mtDNA to hybrid and cybrid progeny (6, 7). For these reasons, we used rhodamine 6G to eliminate autologous mtDNA irreversibly and to generate transmitochondrial myoblasts. We analyzed, at intervals and over an extended time period, the fate of autologous mtDNA in differentiating rhodamine 6G-treated human muscle cultures and myogenic cybrids obtained by fusing rhodamine 6G-treated myoblasts with cytoplasts harboring wild-type or mutated mtDNAs. The resulting transmitochondrial myoblasts were used to quantitate differentiation in normal and respiratory-chain-deficient, transmitochondrial muscle cultures to investigate the contribution of the respiratory chain function in myogenesis.

MATERIALS AND METHODS

Cell cultures. Primary myoblast cultures were obtained from muscle biopsies of patients that were deemed free of muscular disease, as defined by clinical, electrophysiological, morphological, and genetic criteria, whose biopsies were performed in the course of investigation of a disease of the peripheral nervous system. To minimize differences in myoblast growth and differentiation, each set of experiments was performed using muscle derived from the same patient. The cultures were grown as described previously (8, 9).

Three cell lines were used as mitochondrial donors: 143B.TK$^-$, 206/FLP 32.39, and 206/CW 420-115. The wild-type 143B.TK$^-$ cell line contains normally respiring mitochondria (10). The transmitochondrial cell line 206/FLP 32.39 was obtained by fusing mtDNA-less 143B206 ρ^0 cells with enucleated fibroblasts from the Kearns-Sayre syndrome patient K11 (11) harboring a 1,902 bp mtDNA deletion encompassing the region between nucleotides 7846 and 9748 [numbering according to Anderson *et al.* (12)]. The transmitochondrial cell line 206/CW 420-115 was obtained by fusing 143B206 ρ^0 cells with cytoplasts from Kearns-Sayre syndrome patient K13 (11) harboring a 5,790 bp deletion situated between nucleotides 10155 and 15945 of mtDNA. The 206/FLP 32.39 and 206/CW 420-115 cybrids were made homoplasmic for the respective mutations by *in vitro* manipulation of the levels of mutated mtDNA with ethidium bromide (13). They were grown in Dulbecco's modified Eagle's medium (DME) and 10% FBS. The medium for 206/FLP 32.39 and 206/CW 420-115 was supplemented with 50 μg uridine per ml.

The rhodamine 6G-treated myoblasts and the transmitochondrial myogenic cybrids were grown in DME supplemented with 15% FBS, 10 μg insulin per ml, and 50 μg uridine per ml. To induce muscle differentiation, the cells were cultured in mitogen-depleted medium: DME supplemented with 0.5 mg bovine serum albumin per ml, 10 μg insulin per ml, and 50 μg uridine per ml. All culture media contained 10 μg gentamicin per ml and 1 μg amphotericin B per ml. The cultures were grown at saturation humidity in 5% CO_2 and 95% air at 37°C.

Production of transmitochondrial myoblasts. Stock solutions of rhodamine 6G (Eastman Kodak Company, Rochester, NY) were prepared in distilled water (1.0 and 0.1 mg/ml), filter sterilized, and kept at 4°C, protected from light. Clonal myoblasts were exposed to medium containing 4 μg rhodamine 6G per ml for 7 days. Immediately before fusion with enucleated mitochondrial donor cells, the cultures were rinsed with Earle's balanced salt solution, trypsinized, and pelleted by centrifugation. The cytoplasts (see below) and the rhodamine 6G-treated myoblasts were fused by exposure for one min to 50% polyethylene glycol (PEG 1450, American Type Culture Collection, Rockville, MD) prepared in MEM with 10% dimethyl sulfoxide (Sigma, St. Louis, MO) (14).

Cytoplasts were prepared by a modification of the discontinuous Ficoll gradient technique originally described by Wigler and Weinstein (15). Ficoll solutions were prepared in MEM with 1 μg cytochalasin D per ml (Sigma, St. Louis, MO), layered at different concentrations (1.5 ml of 25%, 1.5 ml of 20%, 1.5 ml of 17%, 0.5 ml of 15%, and 0.5 ml of 12.5%) and equilibrated overnight in a CO_2 incubator at 37°C. Approximately 5×10^7 cells were trypsinized and resuspended in 12.5% Ficoll in MEM, containing cytochalasin D. After 30 min they were layered on the gradient and centrifuged at 28,000 rpm in a Beckman SW41 rotor for 1 h at 37°C. The cytoplasts and fragments of cytoplasts harvested from the 12.5%, 15%, and upper portion of the 17% layers were washed three times with MEM and pelleted.

Transmitochondrial myoblasts were cultured in 100 mm dishes. Cells were plated on coverslips in six well-dishes prior to induction of muscle differentiation with mitogen-depleted medium.

DNA analysis. Total DNA was isolated from cell samples collected at distinct time intervals (16). The amount of wild-type mtDNA in myogenic cybrids was quantified as a percentage of total

mtDNA. A series of known quantities of isolated HeLa mtDNA were added to DNA samples of myogenic cybrids. Total cellular mtDNA was quantified by PCR amplification with primers corresponding to the positions 6190–6220 and 7669–7648; $\alpha[^{32}P]$dATP was added to the last amplification cycle. Amplified fragments, digested with Hae III and electrophoresed through 8% polyacrylamide gels, were visualized and quantitated with Bio-Rad Molecular Imager GS363 phosphorimager. The FLP, CW, and autologous myoblast mtDNA derived PCR amplification products digested with Hae III resulted in fragments of 655, 300, 240, 172, 71, and 43 bp, and the Hae III digested HeLa derived PCR products resulted in fragments of 895, 300, 172, 71, and 43 bp. The amount of total mtDNA in DNA samples was determined from the ratio of the 655 + 240 bp FLP, CW, and autologous mtDNA specific fragments to the 895 bp HeLa specific fragment, and from the known concentration of HeLa mtDNA. Specific amplification of wild-type mtDNA was performed with primers corresponding to the positions 9008–9036 and 10542–10518 in the presence of $\alpha[^{32}P]$dATP. The amount of wild-type mtDNA in total DNA samples was determined from Taq I digests of the wild-type specific PCR products, followed by quantitative analysis of the resulting restriction fragments after electrophoresis through 10% polyacrylamide gels. Taq I digestion resulted in fragments of 423, 308, 270, 225, 159, and 107 bp for the amplification products derived from FLP, CW, and autologous myoblast wild-type mtDNAs and fragments of 423, 308, 225, 174, 159, 107, and 96 bp for products derived from HeLa mtDNA. The amount of wild-type mtDNA was derived from the ratio of the 270 bp wild-type specific band to the 174 + 96 bp HeLa specific bands and the known concentration of HeLa mtDNA. The proportion of wild-type mtDNA present in myogenic cybrids was calculated from the ratio of the wild-type mtDNA to the total mtDNA.

Cytochemistry and immunocytochemistry. Cytochrome *c* oxidase (COX) activity was measured cytochemically by the method of Seligman *et al.* (17) as modified by Moraes *et al.* (18). For immunocytochemistry, cells grown on coverslips were fixed in 4% formaldehyde, freshly prepared from paraformaldehyde in PBS (pH 7.4) with 0.1% Triton-X100. Cells were immunostained with antibodies against the lipoyl acetyltransferase (E2) portion of the pyruvate dehydrogenase complex (PDH), which was visualized with a Texas red-labeled goat anti-human antibody. Sarcomeric myosin immunostaining was performed as described by Sancho *et al.* (19). Ethidium bromide at a concentration of 0.05 μg/ml in distilled water was used for the simultaneous detection of nuclei in some experiments.

Myoblast differentiation. The myoblast fusion index was measured by determining the number of nuclei present in myotubes as a percentage of total nuclei in all cells. A modified fusion index was assessed by counting the number of nuclei inside myotubes as a percentage of all nuclei in myosin-positive cells, including mononuclear cells. Myoblast differentiation was also quantitated by determining the ratio of nuclei in myosin-positive mononuclear cells and myotubes as a percentage of the total number of nuclei. At least 200 nuclei from randomly chosen fields were counted in each determination. Results are expressed as the mean of 10 independent determinations. Statistical analysis was performed using analysis of variance and the Tukey test.

RESULTS AND DISCUSSION

Rhodamine 6G treatment. Rhodamine 6G is a lipophilic dye, positively charged at pH 7.0, that selectively accumulates within mitochondria (20). Studies conducted by Gear (21) demonstrated that 3 mM rhodamine 6G strongly inhibits oxidative phosphorylation and ATP-supported ion transport, while 10 μM rhodamine 6G causes uncoupling of oxidative phosphorylation and inhibition of respiration-driven cation transport in isolated mitochondria. The exact mechanism of action of this drug is not yet completely understood. The available evidence suggests that it probably blocks energy transfer in oxidative phosphorylation at two levels: affecting H^+ ejection by redox complexes and inhibiting F1-ATPase (22–24).

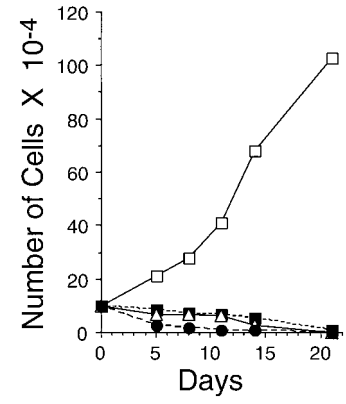

FIG. 1. Growth of myoblasts in the presence or absence of rhodamine 6G. Myoblasts were plated at a density of 1×10^5 cells in multiple 80-cm^2 flasks in 20 ml of growth medium containing rhodamine 6G at 1 μg/ml (solid squares), 4 μg/ml (open triangles), and 16 μg/ml (solid circles) and in rhodamine 6G-free medium (open squares). The culture medium was changed every 3 or 4 days. Cells from individual flasks were trypsinized and counted at the indicated time intervals.

We evaluated the effects of rhodamine 6G on human myoblast proliferation and viability. We performed toxicity studies to identify the optimal concentration of rhodamine 6G needed to block proliferation of human myoblasts. Cell growth was analyzed in cultures treated with 1, 2, 4, 8, 16, and 32 μg rhodamine 6G per ml for up to 21 days. Data from one representative experiment are illustrated in Fig. 1. In the presence of rhodamine 6G, there was a decrease in cell number with time. Microscopic examination of rhodamine 6G-treated cultures revealed that a fraction of the cells became crenated and detached from the growth surface. The detached cells were typically moribund or dead, as determined by the trypan blue dye exclusion test. The vast majority of attached cells were present as single cells rather than clustered in the culture dishes, even after 21 days of culture, indicating that the remaining cells were not mitotically active and were unable to form colonies. At rhodamine 6G concentrations of 8, 16, and 32 μg/ml there were larger numbers of detached cells than at 1, 2, and 4 μg/ml, suggesting that the acute drug toxicity was dose dependent.

To determine whether the rhodamine 6G-induced cytotoxic effects were reversible, the growth and viability of treated myoblasts were monitored by light microscopic inspection of colony formation and by the trypan blue dye exclusion test. Cells plated at a density of 5×10^5 cells per 100 mm plate were treated with 1,

2, 4, 8, 16, and 32 μg rhodamine 6G per ml for 1 to 7 days. After drug treatment, cells were rinsed 3 times in Earle's balanced salt solution and cultured in rhodamine 6G-free medium for 4 weeks. Recovery of growth, as determined by microscopic observation of colony formation in rhodamine 6G-free medium, was observed in some of the myoblast cultures treated with 1 μg rhodamine 6G per ml for 1 to 3 days. However, less than 2 per 10^5 of the plated cells were able to proliferate and colonize after rhodamine 6G treatment. Fewer than 1 per 10^5 of the plated cells were able to proliferate and colonize in the myoblast cultures treated with 2 and 4 μg rhodamine 6G per ml for 6 days. No colonies were observed in cultures treated for 7 days at 1, 2, 4, 8, 16, and 32 μg rhodamine 6G per ml. Scattered, irregularly shaped single cells, some of them rounded or vesiculated, were found in all rhodamine 6G-treated cultures. These small proportions of cells capable of cell growth and forming colonies differ from the expected values for untreated human myoblasts that are 45–60% with these culture conditions. These results indicate that the alterations to cellular function that result from rhodamine 6G treatment are irreversible.

Rescue of rhodamine 6G-treated myoblasts. Since cells treated with rhodamine 6G were not able to proliferate or survive for long periods, we fused rhodamine 6G-treated myoblasts with cytoplasts from other cells to determine whether untreated, exogenous mitochondria could rescue the growth defect and improve viability. The myoblast cultures were exposed to 4 μg rhodamine 6G per ml for 7 days because this concentration was shown to be optimal for preventing subsequent growth of the cells. Treated myoblasts were fused with enucleated cells derived from the 143B.TK$^-$ cell line. Myoblasts from the treated cultures were unable to grow and proliferate. However, the cultures of rhodamine 6G-treated myoblasts that were fused with enucleated 143B.TK$^-$ cells contained numerous cell colonies, all with morphological characteristics of myoblasts. The growth properties of these myoblast cybrids were similar to those of untreated myoblasts (data not shown). Although hybrid formation was not formally excluded in this and subsequent experiments, the growth of morphologically abnormal myoblasts or mitochondrial donor cells was not observed.

To determine whether mitochondrial respiratory chain function was required for the recovery of cell growth, rhodamine 6G-treated myoblasts were also fused with cytoplasts derived from cell lines harboring exclusively mtDNA with large-scale deletions. Because the deleted mtDNA regions encompass respiratory chain subunit genes, as well as tRNA genes that are essential for their translation, these cells exhibit no respiratory chain activity and are not capable of oxidative phosphorylation. Myoblast cultures were treated with 4 μg rhodamine 6G per ml for 7 days, and fused with cytoplasts derived from the 206/FLP 32.39 and 206/CW 420-115 cell lines. These transmitochondrial myogenic cultures contained numerous cell colonies. These myoblasts displayed significant levels of cell growth and continued to proliferate for up to 12 weeks, similar to the treated myoblasts repopulated with wild-type (143B.TK$^-$) mitochondria. These results suggest that the efficiency of recovery of viability was the same for cultures repopulated with wild-type mtDNA as those repopulated with mutated mtDNA. Thus, the introduction of intact mitochondria is sufficient to overcome the growth arrest of cells treated with rhodamine 6G. The absence of ATP generated by oxidative phosphorylation does not appear to impair myoblast viability and proliferation.

Analysis of mtDNA. After repopulation of the rhodamine 6G-treated myoblasts with exogenous mitochondria from the 206/FLP 32.39 and 206/CW 420-115 cell lines, intracellular mtDNA species were analyzed. Only mtDNA molecules with deletions were detected by Southern blotting (data not shown). Quantitative experiments were then performed in samples collected at distinct time intervals, as described under Materials and Methods, to determine whether autologous mtDNA was completely eliminated by rhodamine 6G treatment. Five weeks after PEG fusion with 206/CW 420-115 cytoplasts, 0.18% of total mtDNA in the myogenic cell population consisted of wild-type mtDNA molecules. From the 9th to the 12th week, the proportion of wild-type mtDNA progressively decreased to less than 0.025% of the total mtDNA population. Analysis of the 206/FLP 32.39 derived myogenic cybrids' mtDNA revealed that 5 weeks after PEG fusion only 0.04% of the total mtDNA population was wild-type. From the 9th to the 12th week, the levels of wild-type mtDNA decreased further to 0.005% or less. Thus, the vast majority of the mtDNA of the transmitochondrial myoblasts derived from the 206/FLP 32.39 and 206/CW 420-115 cell lines consisted of mtDNA with a deletion. These levels of autologous mtDNA decreased in amount over time, reinforcing the idea that the loss of endogenous mtDNA is irreversible. The levels of wild-type mtDNA correspond to approximately 1 to 2 mtDNA molecules per myoblast. The low levels of wild-type mtDNA detected in these cell populations may represent mtDNA related sequences that are present in nuclear DNA (25, 26). Alternatively, these residual levels of wild-type mtDNA may result from rare cells in the myoblast populations that retained their autologous mtDNA.

Mitochondria distribution in repopulated myoblasts. Immunocytochemical detection of the mitochondrial enzyme PDH, was performed to evaluate the extent of mitochondrial repopulation of single rhodamine 6G-treated myoblasts in culture, 10 weeks after PEG fusion with 206/FLP 32.39, 206/CW 420-115, or

143B.TK$^-$ cytoplasts. Untreated myoblasts were used as controls. Since this mitochondrial protein is encoded by nuclear DNA, PDH is invariably present in cells with mtDNA mutations, making it a suitable marker to analyze the relative number and distribution of mitochondria in the cell. As illustrated in Fig. 2, PDH immunostaining of the transmitochondrial myogenic cells was similar to that of untreated myoblasts. A punctate staining pattern of mitochondria was present throughout the cell, although mitochondria were more numerous in the perinuclear region. This result indicates that there is a normal number and distribution of mitochondria in the transmitochondrial myoblasts and that the respiratory chain deficiency did not result from a reduced number of organelles or altered protein import into mitochondria.

Respiratory function of rescued rhodamine 6G-treated myoblasts. To examine the respiratory chain function of myogenic cybrids, COX cytochemistry was performed 5 and 13 weeks after PEG fusion. Myoblasts repopulated with 143B.TK$^-$ cytoplasts, thus harboring exclusively wild-type mtDNA, showed levels of COX staining comparable to that of untreated myoblasts. Myogenic cybrids harboring mtDNAs with deletions, thus lacking mitochondrial translation and respiratory chain function, had significantly decreased levels of COX activity, as indicated by the pale background staining for COX activity (Fig. 2). This low level of staining was similar to that observed in ρ^0 cells. Further, no cells with normal COX activity in the myoblast populations were observed, suggesting that rare cells that retained their autologous mtDNA were not present. As expected, the residual levels of wild-type mtDNA that were present in these cultures, $<0.005\%$ of total mtDNA in the FLP myogenic cybrids and $<0.025\%$ in the CW myogenic cybrids, were insufficient to sustain normal respiratory chain activity. It has previously been demonstrated that the threshold for normal respiratory chain function in cultured cells harboring mtDNA with deletions is relatively high, requiring the presence of at least 15% wild-type mtDNA (27, 28).

Myoblast differentiation. During myogenesis, proliferating cells are transformed into highly specialized, multinucleated, contractile muscle fibers. This process is characterized by the irreversible commitment of mononucleated cells to terminal differentiation, loss of proliferative capacity, fusion of myoblasts to form myotubes, induction of muscle-specific genes, and maturation of myotubes into fully functional muscle fibers. Muscle specific myosins are synthesized at an early stage of terminal muscle differentiation, when myoblast fusion normally occurs (29).

To investigate the long-term consequences of deficiencies in oxidative phosphorylation in myogenesis, the capacity of myogenic cybrids to differentiate and fuse to form myotubes and express sarcomeric myosin was quantitated. FLP and CW myogenic cybrids, derived from fusions of rhodamine 6G-treated myoblasts with 206/FLP 32.39 or 206/CW 420-115 cytoplasts, were analyzed for their capacity to fuse and to differentiate. Rhodamine 6G-treated myoblasts fused with 143B.TK$^-$ cytoplasts were used as controls. The cultures were analyzed 1, 2, 3, and 4 weeks after induction of differentiation, corresponding to 10 to 13 weeks after PEG fusion. During the 10 weeks that followed PEG fusion, the cultures were maintained in continuous cultivation to allow myoblast proliferation and to accumulate sufficient number of cells to proceed with the study.

Although the rate of human myoblast differentiation may vary according to several factors, including growth conditions, age of the patient and time in culture, the data obtained for the myogenic cybrids repopulated with wild-type mtDNA were similar to that expected for untreated human myoblasts (9, 30). In both respiration-deficient cultures, the proportion of nuclei inside myotubes (fusion index) was consistently decreased from the first through the fourth week after transfer to mitogen-depleted differentiation medium as compared to controls (Fig. 3A). Myoblast fusion was significantly reduced, ranging from 19% to 50% and from 13% to 28% of the control, respectively for the FLP and CW myogenic cybrids. Similarly, the proportion of nuclei in myotubes compared to the number of nuclei in mononuclear cells and myotubes expressing sarcomeric myosin (modified fusion index) was decreased in respiration-deficient cultures as compared to controls (Fig. 3B). The modified fusion index varied from 47% to 59% and from 33% to 51% of the control for the FLP and CW cultures, respectively. There were no significant differences between the two deficient cultures for both fusion and modified fusion indices.

The distribution of the number of nuclei present in muscle cells expressing sarcomeric myosin (Fig. 4) corroborated the findings of impaired myoblast fusion in respiration-deficient cultures. Despite the deficient fusion capacity, a few myotubes containing 4 or 5 nuclei were observed during the first, second, and third weeks after induction of differentiation in the FLP and CW myogenic cybrids. During the fourth week after induction of differentiation, myotubes containing as many as 9 nuclei could be seen, although rarely, in those cultures (Fig. 4). Further studies are required to determine whether myoblast fusion is primarily affected by a respiratory chain deficiency or secondarily influenced by other phenomena altered by this deficiency. Myoblast fusion involves an active process by which cells migrate, line up in close proximity and remain in contact for a sufficiently long time to allow the lipid bilayers to combine. Therefore, changes affecting any of these steps could account for the observed deficient fusion capacity.

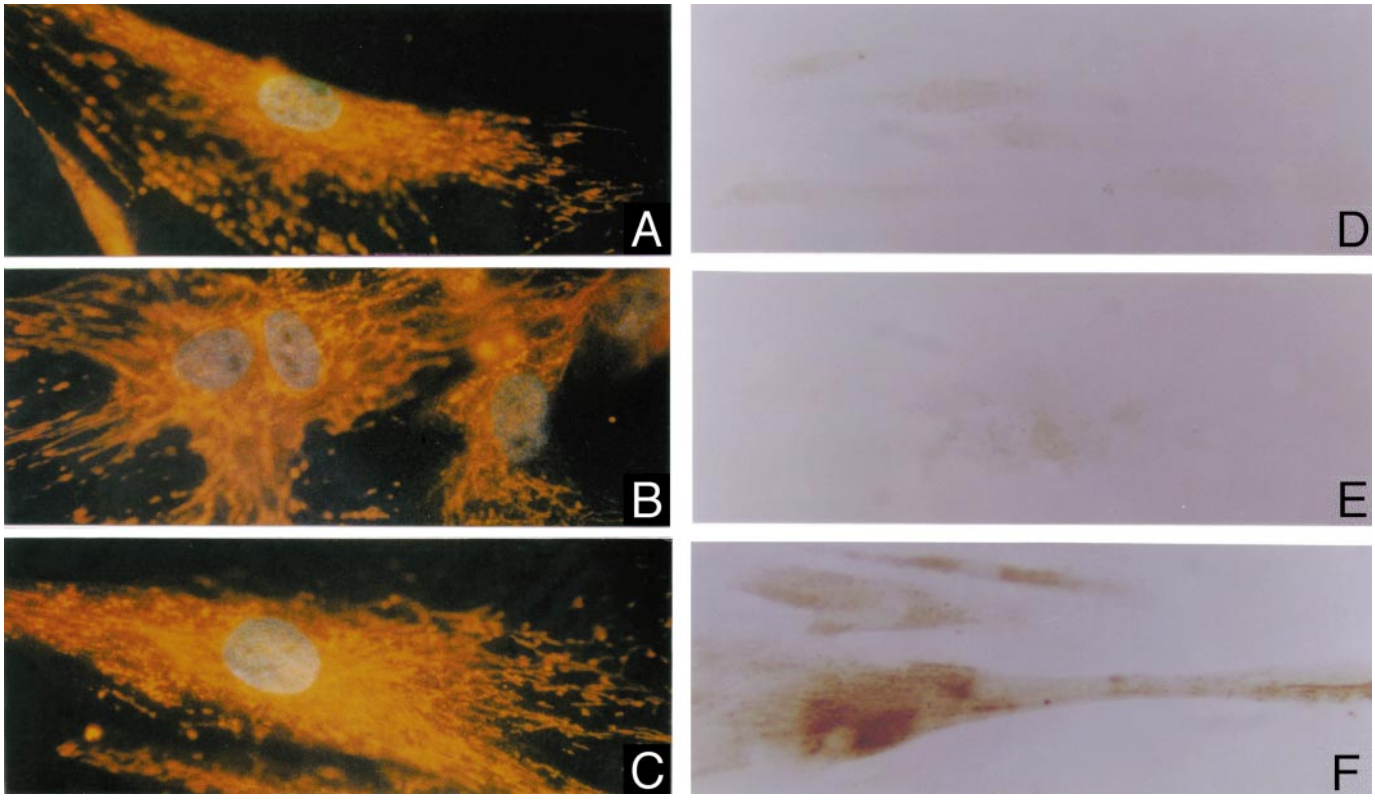

FIG. 2. Localization of mitochondria by PDH immunocytochemistry (A–C) and cytochemical detection of COX activity (D–F) in transmitochondrial myoblasts. Myoblasts treated with rhodamine 6G were fused with cytoplasts derived from 206/FLP 32.39 (A and D) 206/CW 420-115 (B and E), or 143B.TK$^-$ (F). Ten weeks after fusion, the distribution of mitochondria in the cytoplasm of transmitochondrial myoblasts (A and B) was similar to that of untreated myoblasts (C). After 5 weeks in mitogen-depleted medium, no COX activity was detectable in the respiration-deficient myogenic FLP and CW cells (D and E). The levels of COX activity in the 143B.TK$^-$-derived transmitochondrial myoblasts (F) was similar to that of untreated myoblasts (not shown).

The differentiation capacity of myogenic cybrids containing mutated mtDNAs, as determined by the expression of sarcomeric myosin, was also affected (Fig. 3C). During the first 3 weeks in mitogen-depleted "differentiation" medium, the number of nuclei in myogenic cells that expressed sarcomeric myosin was significantly reduced in both FLP and CW myogenic cultures when compared to control cybrids. However, during the fourth week, the values obtained for the FLP myogenic cybrids were similar to the controls. These results indicated that the respiratory chain-deficient cybrid cells were able to differentiate and that this process could be supported entirely by glycolysis. Unfortunately, further evaluation of the cultures derived from the 206/CW 420-115 cell line, to determine whether they would also achieve normal levels of expression of sarcomeric myosin, was not possible, because they showed signs of deterioration, as expected for non-innervated muscle (8).

Conclusions. Rescue of rhodamine 6G-treated cells by fusion with cytoplasts, first demonstrated by Ziegler and Davidson (5) and confirmed by others (6, 7), suggested that introduction of mitochondria could restore cell viability. However, fusion of treated myoblasts with cytoplasts harboring exclusively mtDNA with large-scale deletions (i.e., containing respiratory chain-deficient mitochondria) indicated that restoration of oxidative phosphorylation is not essential for recovery of cell growth and viability. There is evidence by electron microscopic analyses for disruption of mitochondrial membranes in rhodamine 6G-treated cells (31). We hypothesize that the loss of intact endogenous mitochondria is responsible for the loss of mtDNA in rhodamine 6G-treated cells. The introduction of intact, but respiration-deficient mitochondria is sufficient to overcome the growth arrest resulting from rhodamine 6G treatment. Thus, it appears that the loss of intact mitochondria and the subsequent impairment of other metabolic activities that take place within mitochondria are lethal for cells.

The use of rhodamine 6G is particularly advantageous for generating transmitochondrial myoblasts, because, unlike ethidium bromide, treatment for only a few days is required and because mtDNA depletion occurs both in proliferating and non-proliferating cells. Furthermore, the treatment of myoblasts with rhoda-

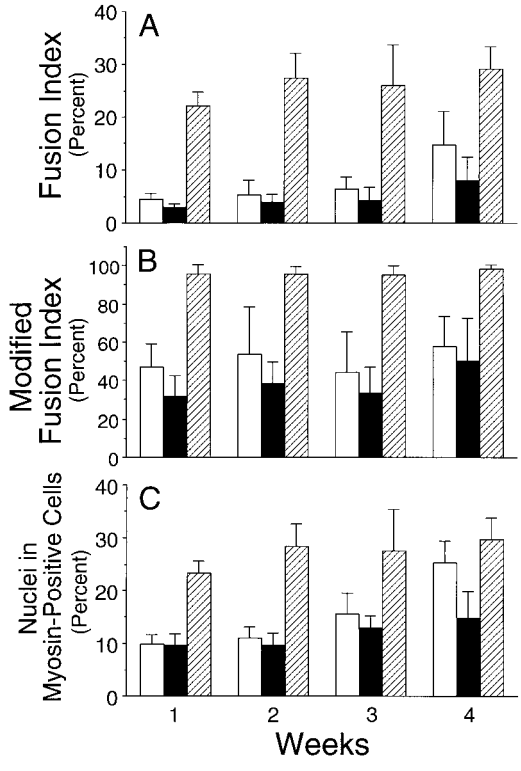

FIG. 3. Fusion (A) and modified fusion (B) indices, and proportion of differentiated myogenic cells (C) of transmitochondrial myoblast cultures at 1, 2, 3, and 4 weeks after induction of muscle differentiation in mitogen-depleted medium. Myoblasts treated with rhodamine 6G were fused with cytoplasts derived from 206/FLP 32.39 (open bar), 206/CW 420-115 (closed bar), or 143B.TK$^-$ cell lines (hatched bar). Myoblast fusion, quantified by both fusion (A) and modified fusion (B) indices, was decreased in FLP and CW-derived transmitochondrial cultures, as compared to transmitochondrial cultures repopulated with wild-type (143B.TK$^-$) mtDNA ($P < 0.001$). Muscle differentiation was also quantitated by determining the number of nuclei in all myosin-positive cells as a percentage of the total number of nuclei in each culture. The difference between the respiration-deficient FLP and CW-derived myogenic cultures and wild-type 143B.TK$^-$-derived cultures are significant at 1, 2, and 3 weeks after induction of differentiation ($P < 0.001$) (C). At 4 weeks, however, the percentage of nuclei in sarcomeric myosin-positive FLP-derived myogenic cultures was similar to the wild-type 143B.TK$^-$-derived cultures. Muscle differentiation in the respiratory-chain-deficient CW-derived myogenic cultures was still significantly lower in the fourth week ($P < 0.001$) (C). Error bars represent $\pm$ 1 SD.

mine 6G eliminates the need for selectable markers to obtain homogeneous cybrid populations, because rhodamine 6G-treated myoblasts do not proliferate and eventually die, unless they are repopulated with exogenous mitochondria. Because the cells cannot resume growth after removal of rhodamine 6G from the medium, the disruption of the mitochondrial membrane by rhodamine 6G is apparently an irreversible process.

In this study, we use a well defined, post-mitotic *in vitro* system to investigate whether myogenesis is affected by deficiencies in oxidative phosphorylation. This system is based upon fusion of rhodamine 6G-treated human myoblasts with cytoplasts to create transmitochondrial muscle cells that are capable of differentiation. We demonstrate that rhodamine 6G-treated myoblasts can be rescued with cytoplasts derived from patients with pathogenic mtDNA deletions, to introduce mtDNA with defined mutations into these cells. Long-term quantitative analysis of muscle cells repopulated with mtDNA with deletions indicated that there was impairment of the fusion of myoblasts to form multinucleated syncytia. Differentiation, as defined by the expression of sarcomeric myosin, was also delayed in respiratory chain-deficient cultures. However, myogenic differentiation was not indefinitely compromised, since the levels of differentiation recovered to wild-type levels in some respiratory chain deficient cell lines during extended culture times. These results confirm the importance of oxidative phosphorylation as a source of ATP for muscle development, but also demonstrate that myoblast fusion and differentiation can be supported by glycolysis, even in the complete absence of respiratory chain function.

Further applications for this cell culture model include longer-term studies of the consequences of mitochondrial dysfunction on the differentiation and function of muscle in aneural and innervated myoblast cultures. The use of rhodamine 6G to create transmitochondrial cells allows the selective elimination of the endogenous mitochondrial elements and repopulation of cells with exogenous mitochondria containing wild-type or mutated mtDNA. This method is particularly useful for non-proliferating cultures bound towards terminal differentiation. In this way, specific mtDNA mutations can be introduced into myoblasts, as well as into primary cells or established cell lines derived from other tissues. Myoblasts are capable of differentiation into multinucleated myotubes, which can be innervated with fetal rat spinal cord neurons (32, 8). The

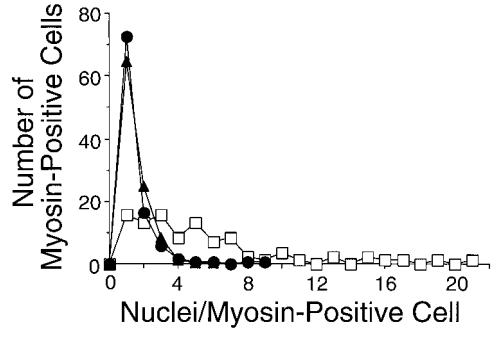

FIG. 4. The distribution of the number of nuclei present in mononuclear cells and multinucleated myotubes expressing sarcomeric myosin. The number of nuclei inside cells and myotubes expressing sarcomeric myosin in the 206/FLP 32.39 (closed triangles) and 206/CW 420-115 (closed circles) derived myogenic cultures is significantly less than the wild-type 143B.TK$^-$ (open squares) derived myogenic cultures 4 weeks after induction of differentiation, further confirming impaired myoblast fusion in the respiration-deficient cultures.

resulting mature, functionally active myofibers are viable for months, enabling the study of the long-term consequences of mtDNA mutations and respiratory chain deficiency on muscle differentiation and contractile function.

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REFERENCES

1. Attardi, G., and Schatz, G. (1988) *Annu. Rev. Cell Biol.* **4,** 289–333.
2. Boulet, L., Karpati, G., and Shoubridge, E. A. (1992) *Am. J. Hum. Genet.* **51,** 1187–1200.
3. Herzberg, N. H., Zwart, R., Wolterman, R. A., Ruiter, J. P. N., Wanders, R. J. A., Bolhuis, P. A., and van den Bogert, C. (1993) *Biochim. Biophys. Acta* **1181,** 63–67.
4. DiMauro, S., and Moraes, C. T. (1993) *Arch. Neurol.* **50,** 1197–1208.
5. Ziegler, M. L., and Davidson, R. L. (1981) *Somat. Cell Genet.* **7,** 73–88.
6. Huston, M. M., Smith, III, R., Hull, R., Huston, D. P., and Rich, R. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3286–3290.
7. Trounce, I., and Wallace, D. C. (1996) *Somat. Cell Mol. Genet.* **22,** 81–85.
8. Miranda, A. F., Peterson, E. R., and Masurovsky, E. B. (1988) *Tissue Cell* **20,** 179–191.
9. Grubic, Z., Komel, R., Walker, W. F., and Miranda, A. F. (1995) *Neuron* **14,** 317–327.
10. King, M. P., and Attardi, G. (1989) *Science* **246,** 500–503.
11. Mita, S., Rizzuto, R., Moraes, C. T., Shanske, S., Arnaudo, E., Fabrizi, G., Koga, Y., DiMauro, S., and Schon, E. A. (1990) *Nucleic Acids Res.* **18,** 561–567.
12. Anderson, S., Bankier, A. T., Barrell, B. G., Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) *Nature* **290,** 457–465.
13. King, M. P. (1996) *Methods Enzymol.* **264,** 339–344.
14. Davidson, R. L., and Gerald, P. S. (1977) *Methods Cell Biol.* **15,** 325–338.
15. Wigler, M. H., and Weinstein, I. B. (1975) *Biochem. Biophys. Res. Commun.* **63,** 669–674.
16. Davis, L. G., Dibner, M. D., and Battey, J. F. (1986) Methods in Molecular Biology, Elsevier, New York.
17. Seligman, A. M., Karnovsky, M. J., Wasserkrug, H. L., and Hanker, J. S. (1968) *J. Cell Biol.* **38,** 1–14.
18. Moraes, C. T., Schon, E. A., DiMauro, S., and Miranda, A. F. (1989) *Biochem. Biophys. Res. Commun.* **160,** 765–771.
19. Sancho, S., Mongini, T., Tanji, K., Tapscott, S. J., Walker, W. F., Weintraub, H., Miller, A. D., and Miranda, A. F. (1993) *N. Engl. J. Med.* **329,** 915–920.
20. Huang, C.-H. (1969) *Biochemistry* **8,** 344–352.
21. Gear, A. R. L. (1974) *J. Biol. Chem.* **249,** 3628–3637.
22. Higuti, T., Niimi, S., Saito, R., Nakasima, S., Ohe, T., Tani, I., and Yoshimura, T. (1980) *Biochim. Biophys. Acta* **593,** 463–467.
23. Wieker, H.-J., Kuschmitz, D., and Hess, B. (1987) *Biochim. Biophys. Acta* **892,** 108–117.
24. Bullough, D. A., Ceccarelli, E. A., Roise, D., and Allison, W. S. (1989) *Biochim. Biophys. Acta* **975,** 377–383.
25. Hirano, M., Shtilbans, A., Mayeux, R., Davidson, M. M., DiMauro, S., Knowles, J. A., and Schon, E. A. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14894–14899.
26. Wallace, D. C., Stugard, C., Murdock, D., Schurr, T., and Brown, M. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14900–14905.
27. Hayashi, J.-I., Ohta, S., Kikuchi, A., Takemitsu, M., Goto, Y.-I., and Nonaka, I. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10614–10618.
28. Sancho, S., Moraes, C. T., Tanji, K., and Miranda, A. F. (1992) *Somat. Cell Mol. Genet.* **18,** 431–442.
29. Franzini-Armstrong, C., and Fischman, D. A. (1994) *in* Myology (A. G. Engel and C. Franzini-Armstrong, Eds.), McGraw-Hill, New York.
30. Fisher, P. B., Miranda, A. F., Mufson, R. A., Weinstein, L. S., Fujiki, H., Sugimura, T., and Weinstein, I. B. (1982) *Cancer Res.* **42,** 2829–2835.
31. Zhang, W. W., Hood, R. D., and Smith-Sommerville, H. E. (1990) *Toxicol. Lett.* **51,** 35–40.
32. Kobayashi, T., and Askanas, V. (1985) *Exp. Neurol.* **88,** 327–335.