Status of Mitochondria in Living Human Fibroblasts during Growth and Senescence in Vitro:
Use of the Laser Dye Rhodamine 123

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ABSTRACT Rhodamine 123, a fluorescent laser dye that is selectively taken up into mitochondria of living cells, was used to examine mitochondrial morphology in early-passage (young), late-passage (old), and progeric human fibroblasts. Mitochondria were readily visualized in all cell types during growth (mid-log) and confluent stages. In all cell strains at confluence, mitochondria became shorter, more randomly aligned, and developed a higher proportion of bead-like forms. Treatment of cells for six days with Tevenel, a chloramphenicol analog that inhibits mitochondrial protein synthesis, brought about a marked depletion of mitochondria and a diffuse background fluorescence. Cyanide produced a rapid release of preloaded mitochondrial fluorescence followed by detachment and killing of cells. Colcemid caused a random coiling and fragmentation of mitochondria particularly in the confluent stage. No gross differences were discernible in mitochondria of the three cell strains in mid-log and confluent states or after these treatments.

Butanol-extractable fluorescence after loading with rhodamine 123 was lower in all cell strains in confluent compared to mid-log stages. At confluence all three cell strains had similar rhodamine contents at zero-time and after washout up to 24 h. At the mid-log stage, young cells contained more rhodamine initially and lost it more rapidly than old or progeria cells, in that order.

The data indicate no gross derangement in the morphology or number of mitochondria in old and progeria fibroblasts but there is a reduction of protonmotive force evident in these cells at the mid-log stage that may be growth limiting.

Mitochondria play a central role in the energy metabolism of virtually all living cells (22). These intracellular organelles are morphologically dynamic such that their shape and distribution can be affected by the state of metabolism, proliferation, differentiation, as well as pathological involvement (12, 13, 26-28, 35). Because many of these states can be readily altered in cell culture, this system provides an excellent opportunity to study mitochondrial plasticity. A further advantage is that cultured human fibroblasts can be derived from virtually all persons, enabling the study of mitochondria from any given subject. Moreover, cultured human fibroblasts have a limited replicative lifespan that provides an excellent model for the study of biological aging (10, 16). Several mechanisms have been proposed for the loss of replicative capacity, among them the possibility of failure in energy metabolism consequent to depletion or derangement of mitochondria (10, 16). Earlier studies on energy metabolism in human fibroblasts have indicated no change in mitochondrial function during serial passage (5, 14). But more recent work (Goldstein et al., manuscript submitted for publication) has revealed certain metabolic patterns consistent with a mitochondrial deficit in late-passage cells, and this has been borne out by electron microscopy studies showing a decrease in the number and quality of mitochondria (18, 23, and Goldstein et al., manuscript in preparation). However, electron microscopy, of necessity, involves the use of fixed preparations and therefore gives no information on functional correlates to morphology.

Recently, Johnson et al. (19) have described the use of the
laser dye rhodamine 123 to stain mitochondria directly and selectively, i.e. without passage through endocytic vesicles and lysosomes, in a variety of cultured cells from lower forms. The mechanism for this specific staining has not yet been determined but likely involves the positive charge of the dye at physiologic pH and the attraction of the relatively high negative electrical potential across the mitochondrial membrane (19, 20). Thus this method provides a unique opportunity not only to examine mitochondria in the living state but also to obtain quantitative information on the relative protonotive force of the mitochondria under various conditions (1, 7).

The present study was undertaken to observe mitochondrial morphology and to measure uptake and release of rhodamine 123 in cultured human fibroblasts during growth from sparse to confluent stages and after treatment with various chemical agents. We compared early-passage (young) and late-passage (old) normal skin fibroblasts, and one cell strain from a subject with the Hutchinson-Gilford (progeria) syndrome (9). The latter strain was included because progeria subjects undergo premature aging and have an increase in total body O\textsubscript{2} consumption (32) and muscle mitochondrial oxidation (33) whereas cultured progeric fibroblasts show an abbreviated replicative lifespan (see reference 9) and evidence of decreased cell respiration (Goldstein et al., manuscript submitted for publication).

MATERIALS AND METHODS

Cell Culture

Human skin fibroblasts were established and grown by standard methods (11) in Eagle's minimum essential medium plus nonessential amino acids, 1 mM pyruvate, and 10% fetal calf serum. The cell strain A2 was derived from a normal 11-yr-old male and had a replicative lifespan of 65 mean population doublings (MPD) (15). The progeria strain (P18) was derived from a 5-yr-old female with the classical disease and had a curtailed replicative lifespan, compared to age-matched controls, of 42 MPD (9). All experiments on young fibroblasts were carried out in the first half of the replicative lifespan when cells required ~6 d to become confluent after a 1:8 split. Old cells were used when between 75 to 90% of the lifespan was completed. Because of their slower growth they were split at 1:4 and required 6–8 d to become confluent. Progeria cells were used in the first half of their lifespan and were split at 1:4. They were intermediate in growth vigor to young and old cells and required 5–6 days to become confluent. Mid-log cells were studied about halfway through their growth interval when cells occupied about half of the available growth surface as observed microscopically. Confluent cells were studied on the first or second day after they covered the growth surface.

Rhodamine 123 Staining

Cells were prepared for staining as described by Johnson et al. (19) with slight modifications. In brief, cells were grown on 12 mm round cover slips in regular growth medium and treated either directly with rhodamine 123 or after exposure to various agents as described below. Rhodamine 123 (Eastman Laboratory and Specialty Chemicals, Rochester, N. Y.), a gift of Dr. Lincoln Johnson, was made up as a stock solution in dimethyl sulfoxide at 1 mg/ml and kept at 4°C in the dark. The stock solution was made up fresh every 2 wk. Immediately before use rhodamine 123 was diluted in growth medium to 10 μg/ml and added to cells that were incubated in a 95%-air, CO\textsubscript{2} incubator at 37°C for 30 min. Cover slips were then rinsed through three 5-ml changes of medium, 5 min per rinse, and mounted in regular growth medium on a live-cell observation chamber fashioned from silicone rubber punched with 10-mm holes and pressed onto a standard 25 x 75 mm microscope slide. Stained cells were examined by epifluorescent illumination on a Wild-Leitz Orthomat fluorescence microscope equipped with a Philips CS 100 W-2 mercury lamp, fluorescein isothiocyanate (FITC) 490 nm and K510 nm suppression filters and a 4-mm BG 38 exciting filter. Slides were placed on a Leitz heated stage (500-117,006) at 37°C ± 0.5°C throughout microscopic examination. Photographs were made using Kodak Tri X (ASA 400) and film exposure times ranged from 9-12 s.

Treatment of Cells with Chemical Agents

Tevenel, the sulfamoyl analog of chloramphenicol, was added to cultures after a 1:4 split and cells were allowed to grow to confluence over two mean population doublings (MPD). Cells were then stained as described above with rhodamine 123. Cyanide, because of its rapid action, was added after cells were stained with rhodamine 123 just before microscopic observation. Colcemid was added at 10 μg/ml for 16 h followed by staining of the cells with rhodamine 123.

Quantitation of Butanol-extractable Rhodamine 123

Cells were subcultured into 60-mm plastic petri dishes containing regular growth medium. At either confluent or mid-log phases, the medium was removed and 2 ml of growth medium containing 10 μg/ml rhodamine 123 was added. Cells were then incubated for 30 min at 37°C. The rhodamine-containing medium was then removed by suction and rinsing was done by adding 5 ml of fresh growth medium and incubating for 5 min at 37°C in the standard incubator. This was repeated two more times. After the third rinse, zero-time fluorescence was determined along with fluorescence at subsequent time-points at 2, 6, and 24 h in replicate dishes. Appropriate blanks (cells not treated with rhodamine and cell-free dishes treated with rhodamine) were also run simultaneously. At each time-point, medium was removed by suction and dishes were rinsed twice with phosphate-buffered saline. 1 ml of isobutanol was then added to each dish for 5 min and removed, followed by an additional 0.5 ml of isobutanol and pooling with the first extract. Standard curves were determined in each experiment from 1–200 nM rhodamine 123 and found to be consistently linear over this range of concentrations. Percent fluorescence was determined on an Amino-Bowman spectrophotofluorometer with settings at 508-nm excitation and 536-nm emission. Fluorescence of blank controls, which was always < 10% of the fluorescence of test dishes containing cells treated with rhodamine, was subtracted in each case. Cell protein was determined by the method of Lowry et al. (24) on replicate dishes.

RESULTS

Effect of Growth State on Mitochondrial Staining Patterns

Mitochondria stained by rhodamine 123 were easily discerned in all three cell types during mid-log phase (Fig. 1 a–c) and during confluent phase (Fig. 1 d–f). In general, the nuclear area was devoid of fluorescent staining and this produced a darkened round-to-oval image. There was a trend during growth of cells to confluence for an increasing proportion of mitochondria to become shorter, more randomly aligned, and to take on beadlike forms. Although this was best seen in young confluent cells (Fig. 1 d) these effects were also visible in sparse cultures of all cell types if two or more cells came into contact. Due to considerable heterogeneity in cell size and shape plus rapid fading of fluorescence on illumination, we did not attempt to quantify the mitochondrial fluorescence in situ. Moreover, a substantial intercellular range of mitochondrial fluorescence was seen even in cells of comparable size and shape. However, there did not appear to be any gross differences between young, old, and progeria cells during mid-log or confluent stages (see below). Further, in no strain did we find individual cells that appeared severely depleted of mitochondria.

Effect of Tevenel

Tevenel, the sulfamoyl analog of chloramphenicol, inhibits mitochondrial protein synthesis (8). After 6 d of treatment of cells over two mean population doublings, Tevenel should, therefore, deplete various mitochondrial protein components and render these organelles less able to develop and maintain functional competence. This was readily apparent (Fig. 2) as the effect of Tevenel was to decrease the number of discretely stained mitochondria and produce a faint diffuse background fluorescence. This occurred to nearly the same extent in all three cell types (not shown).
Effect of Cyanide

Treatment of cells for 10 min with $10^{-3}$ M cyanide brought about a rapid dispersion of fluorescence from the mitochondria, resulting in low-level, diffuse cytoplasmic fluorescence whether in sparse (Fig. 3 a) or confluent states (Fig. 3 b). This effect was reversible as mitochondria of cells rinsed free of cyanide could be restained with rhodamine. No obvious difference was found in the response of young, old, or progeria cells to cyanide at $10^{-3}$ M, and, in all strains, longer times of exposure (hours rather than minutes) to cyanide brought about total cellular detachment. However, it was noted that $10^{-4}$ M cyanide could disperse the mitochondrial fluorescence in young cells but was without effect in old and progeria cells.
all three cell types contained the same amount of residual fluorescence at a faster rate than old and progeria cells, so that at 24 h after loading with rhodamine 123, followed by up to 24 h of washout. However, the initial rhodamine content was equal in all three cell types at confluence, both at zero-time and during 24 h of washout. However, the initial rhodamine content of all cell types was lower in confluent than in mid-log cells. Further, in the mid-log stage young cells accumulated more rhodamine at zero-time than old cells and progeria cells. This suggests, in concert with the findings in phytohemagglutinin-stimulated lymphocytes (6), that the number of mitochondria-specific fluorescence of several kinds of cultured mammalian cells (20). Our results, therefore, are best explained by a gradual depletion of critical mitochondrial proteins, bringing about dissolution of mitochondria and greater leakiness, respectively. Short-term treatment of cells with cyanide produced a sudden release of fluorescence, probably due to its effect as a rapid poison of cytochrome oxidase and hence of respiration (22). It is noteworthy that other inhibitors of electron transport, and ionophores that decrease mitochondrial membrane potential, had similar effects. Colcemid treatment of cells led to a pronounced distortion of mitochondrial morphology, likely because this agent depolymerizes microtubules that are involved in the maintenance of mitochondrial shape, distribution, and migration (17, 19, 30, 31, 34).

Although no gross morphological differences between the mitochondria of the three cell types were apparent either with respect to the stage of growth or after treatment with the various agents, results on butanol-extracted rhodamine fluorescence were of great interest. Initial rhodamine content was highest at the time of vigorous DNA synthesis and mitosis. It further implies that the trend we observed toward shorter, more randomly aligned mitochondria with a greater proportion of bead-like forms in confluent cells is accompanied by a state of reduced oxidative phosphorylative activity and hence lower proton motive force. Indeed, this is supported by electron microscopy studies (2, 4, 25) showing a decrease in mitochondrial mass, and metabolic studies (Goldstein et al. Submitted for publication.) showing decreased O2 consumption.

**DISCUSSION**

Use of the laser dye rhodamine 123 enables the selective staining of mitochondria in living animal cells and particularly good mitochondrial visualization in the flattened cultured fibroblast. The mechanism for selective uptake of this dye into mitochondria has not yet been confirmed but likely resides in the attraction of the cationic rhodamine 123 molecule toward the relatively high electronegative potential that exists across the mitochondrial membrane (1, 7, 19, 20). This method, therefore, provides a means not only to visualize mitochondria of living cells directly but also to assess total proton motive force within the mitochondrial mass and compare different human fibroblast strains under various conditions.

Clearly apparent on microscopic examination was a marked cell-to-cell variability in fluorescence. Such heterogeneity was also reported recently for a variety of cultured mammalian cells including human fibroblasts (20) and for phytohemagglutinin-stimulated human lymphocytes (6). In the latter case, as in our study, it was also observed that maximal rhodamine uptake occurred coincident with peak DNA synthesis and mitosis.

Tevenel, a chloramphenicol analog that inhibits mitochondrial protein synthesis (8), reduced the number of mitochondria after 6 d and rendered many of those surviving less avid in trapping and holding the dye. It is of interest that chloramphenicol treatment for 24 h had no discernible effect on mitochondria-specific fluorescence of several kinds of cultured mammalian cells (20). Our results, therefore, are best explained by a gradual depletion of critical mitochondrial proteins, bringing about dissolution of mitochondria and greater leakiness, respectively. Short-term treatment of cells with cyanide produced a sudden release of fluorescence, probably due to its effect as a rapid poison of cytochrome oxidase and hence of respiration (22). It is noteworthy that other inhibitors of electron transport, and ionophores that decrease mitochondrial membrane potential, had similar effects. Colcemid treatment of cells led to a pronounced distortion of mitochondrial morphology, likely because this agent depolymerizes microtubules that are involved in the maintenance of mitochondrial shape, distribution, and migration (17, 19, 30, 31, 34).

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FIGURE 3 Mitochondrial staining by rhodamine 123 after treatment of live human fibroblasts with cyanide. Young A2 cells were prepared in the usual manner during mid-log (a) or confluent (b) stages. Cells were then exposed for 10 min to cyanide $10^{-3}$ M and mounted as in Materials and Methods (for fluorescence microscopy). The 10 min includes the time of first cyanide exposure to the time of photography.

when cells are confluent.

The more rapid rate of loss of rhodamine 123 from young mid-log cells, however, appears paradoxical. Among the possible explanations is a higher rate of mitochondrial turnover and hence more rapid release of intramitochondrial rhodamine 123 during vigorous cell division. Alternatively, there could be an active mechanism that first traps this cationic dye more avidly in dividing cells and then more actively secretes it. Indeed, cells have a mitochondrial pump dependent on respiration which is the prime determinant of influx into and efflux from mitochondria of various naturally occurring cations (3, 21, 29).

Finally, although the data indicate a reduction of mitochondrial functional competence in old normal and progeria cells that appears in relatively sparse cultures (when cells would normally undergo vigorous cell division), we cannot conclude that this is causal to the restricted growth capacity of these two cell types. Mitochondrial function is only moderately reduced and it does, in fact, rise at mid-log compared to the confluent state. On the other hand, perhaps the inability of old and progeria cells to augment mitochondrial mass and/or proton-motive force to the same extent as young cells becomes growth limiting. That progeria fibroblasts have a reduced ability to trap rhodamine in the face of their decreased respiration (Goldstein et al., manuscript submitted for publication), clearly implies that progeric cells are less efficient bioenergetically than young or old normal cells. Further studies are needed to define the relationship between mitochondrial function and the abbreviated lifespan of cultured progeria cells and the loss of replicative capacity in normal cells after repeated serial passage.

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FIGURE 4 Mitochondrial staining of live human fibroblasts by rhodamine 123 after treatment with colcemid. Young A2 cells were incubated for 16 h with colcemid 10 μg/ml and then immediately prepared for mitochondrial staining and photography. Panel a: mid-log cells; Panel b: confluent cells.

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