Nuclear-recessive Mutations of Factors Involved in Mitochondrial Translation Are Responsible for Age-related Respiration Deficiency of Human Skin Fibroblasts*

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We addressed the question of whether both mitochondrial and cytoplasmic translation activities decreased simultaneously in human skin fibroblasts with the age of the donors and found that the age-related reduction was limited to mitochondrial translation. Then, to determine which genome, mitochondrial or nuclear, was responsible for this age-related, mitochondria-specific reduction, pure nuclear transfer was carried out from mitochondrial DNA (mtDNA)-less HeLa cells to four fibroblast lines, two from aged subjects, one from a fetus, and one from a patient with cardiomyopathy, and their nuclear hybrid clones were isolated. A normal fibroblast line from the fetus and a respiration-deficient fibroblast line from the patient were used as a positive and a negative control, respectively. Subsequently, the mitochondrial translation and respiration properties of the nuclear hybrid clones were compared. A negative control experiment showed that this procedure could be used to isolate even nuclear hybrids expressing overall mitochondrial respiration deficiency, whereas no respiration deficiencies were observed in any nuclear hybrids irrespective of whether their mtDNAs were exclusively derived from aged or fetal donors. These observations suggest that nuclear-recessive mutations of factors involved in mitochondrial translation but not mtDNA mutations are responsible for age-related respiration deficiency of human fibroblasts.

It has been presumed that somatic mutations accumulate in mitochondrial DNA (mtDNA) much faster than in nuclear DNA because mitochondria are highly oxygenic organelles due to their function in producing energy, mtDNA lacks histones protecting it from mutagenic damage, and its repair systems are limited (1). Therefore, it has been proposed that the accumulation of various somatic mutations in mtDNA and the resultant decrease in mitochondrial respiratory function could be involved in aging processes in mammals (2–4). There have been many reports that the respiration capacity of mitochondria in highly oxidative tissues decreases during aging (4). Moreover, the accumulation of somatic and pathogenic mtDNA mutations, which have been shown to cause various kinds of mitochondrial encephalomyopathies (5–8), was also shown to increase with age in normal subjects (9, 10). However, as the nuclear genome encodes most mitochondrial proteins including factors necessary for replication and expression of the mitochondrial genome, it is possible that only mutations in the nuclear genome contribute to the age-related decline of mitochondrial respiratory function. In fact, there is no convincing evidence that mtDNA somatic mutations are responsible for this age-related phenotype.

Previously, we observed age-related reduction of cytochrome c oxidase (COX1) activity and mitochondrial translation in cultured human skin fibroblasts isolated from donors of various ages (0–97 years), and in studies on their mtDNA transfer to mtDNA-less (ρ0) HeLa cells, we showed that mtDNA mutations were not responsible for the observed age-related mitochondrial dysfunction of human skin fibroblasts (11). Recently, using an mtDNA transfer system similar to ours (11), i.e. mtDNA transfer from human skin fibroblasts to ρ0 human cells, Laderman et al. (12) reported contradictory results, suggesting the presence of age-related heritable alterations in fibroblast mtDNA. However, the procedure for isolating cybrids by the transfer of fibroblast mtDNA to ρ0 cells is not appropriate for unambiguous determination of whether accumulation of mtDNA mutations is involved in age-related mitochondrial dysfunction, because during selection to exclude ρ0 cells using medium without uridine and/or pyruvate, cybrids expressing respiration deficiency due to accumulation of mtDNA mutations might also be eliminated preferentially, so that only cybrids with normal mitochondrial respiratory function are isolated. Thus in these conditions (11, 12), defective cybrids with mutant mtDNA from both young and aged subjects may have been excluded preferentially.

Recently, to determine whether the mitochondrial or nuclear genome is responsible for mitochondrial diseases, we developed a system for delivery of a normal nuclear genome from ρ0 HeLa cells to fibroblasts from patients with respiration deficiency by isolating nuclear hybrids (13). In these nuclear hybrid clones, the nuclear genome was derived from both parental ρ0 HeLa cells and fibroblasts, whereas the mitochondrial genome is exclusively from fibroblasts. Thus, if mitochondrial dysfunction is restored by the introduction of pure ρ0 HeLa nuclei, it should be attributed to nuclear-recessive mutations, and if not, it should be due to either mtDNA mutations or nuclear-dominant mutations. Moreover, since HeLa nuclei completely free from

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1 The abbreviations used are: COX, cytochrome c oxidase; HAT, hypoxanthine/aminopterin/thymidine; Oua, ouabain; TIG, Tokyo Metropolitan Institute of Gerontology; PCR, polymerase chain reaction; bp, base pair(s).
mitochondrial defects—Telomere shortening and telomerase activity could be due at least partly to reduction of mitochondrial translation.

**RESULTS**

First, we compared the COX activities of three fibroblast lines from fetuses and six from aged subjects (70, 72, 80, 81, 86, and 97 years old) and confirmed that the COX activities of the lines from aged subjects were only about 20–40% of those of the fetal lines (Fig. 1), consistent with our previous observations (11). To determine the reasons for the decrease in COX activity in aged subjects, we compared the levels of mtDNA and its transcripts in the fibroblasts. Southern blot and Northern blot analyses showed that their levels did not change substantially with the age of the fibroblast donors (Fig. 2). Then we compared the mitochondrial translation activities of the fibroblasts by measuring [35S]methionine incorporation into mtDNA-encoded polypeptides in the presence of emetine to inhibit mitochondrial translation. Results showed that mitochondrial translation activity decreased with aging (Fig. 3a). In contrast, we found that the cytoplasmic translation activity in fibroblasts remained constant, as shown by [35S]methionine incorporation into nuclear DNA-encoded polypeptides. These results suggest that age-related reduction of translation activity is limited to mitochondria. Therefore, the observed age-related decrease of COX activity could be due at least partly to reduction of mitochondrial translation.
Since mutations in both nuclear and mitochondrial genomes contribute to reduction of mitochondrial translation activity (21, 22), we examined which genome was responsible for the age-related, mitochondria-specific reduction. For this, we used our recently developed procedure for delivery of pure normal nuclear genomes from $\text{r}^0$ HeLa cells to fibroblasts by isolating nuclear hybrids (13). As $\text{r}^0$ HeLa cells have been shown to possess no mtDNA (6) and to be resistant to both 6-thioguanine and Oua, HeLa nuclei free from mtDNA could be introduced into fibroblasts simply by fusion of fibroblasts with $\text{r}^0$ HeLa cells followed by cultivation in selective medium with Oua HAT (Table I). Oua and HAT were used to exclude unfused parental fibroblasts and $\text{r}^0$ HeLa cells, respectively (see “Materials and Methods”). Accordingly, the nuclear genome of the nuclear hybrids was derived from both parental $\text{r}^0$ HeLa cells and fibroblasts, whereas the mitochondrial genome was exclusively from fibroblasts.

First, it was necessary to show unambiguously that our nuclear genome delivery system did not exclude clones expressing complete respiration deficiency, even though our system does not have to use selective pressure upon mitochondrial respiratory function for removal of parental $\text{r}^0$ HeLa cells. To demonstrate the reliable isolation of respiration-deficient clones, we carried out nuclear transfer from $\text{r}^0$ HeLa cells to respiration-deficient fibroblasts containing 90% mtDNA with a pathogenic mutation (A to G) in tRNA$_{\text{Ile}}$ at 4,269 derived from a patient with fatal cardiomyopathy (14, 15). Then we examined the content of the mtDNA with the tRNA$_{\text{Ile}}$ 4,269 mutation in all 12 nuclear hybrid clones by analysis of the SspI restriction pattern of the PCR products as described previously (15). The results showed that seven nuclear hybrid clones contained more than 95% mutant mtDNA (Table I). We compared mitochondrial translation activity by $[^{35}\text{S}]$methionine incorporation into clones containing predominantly the mutant or wild-type mtDNA. Fig. 4 shows that $[^{35}\text{S}]$methionine incorporation into
Somatic cell genetic characteristics of parent cells and their nuclear hybrids

| Cell lines       | Drug resistance | Cross             | Selection | Number of clones |
|------------------|-----------------|-------------------|-----------|------------------|
| Parent cell lines|                 |                   |           |                  |
| Nuclear donor    |                 |                   |           |                  |
| ρ0 HeLa          |                 |                   |           |                  |
| Nuclear recipients|               |                   |           |                  |
| TIG3S (80)       |                 |                   |           |                  |
| TIG106 (80)      |                 |                   |           |                  |
| CM (18)          |                 |                   |           |                  |
| Nuclear hybrids  |                 |                   |           |                  |
| NH3S             |                 | ρ0HeLa × TIG3S    | Oua + HAT | 12               |
| NH106            |                 | ρ0HeLa × TIG106   | Oua + HAT | 12               |
| NH102            |                 | ρ0HeLa × TIG102   | Oua + HAT | 12               |
| NHCM             |                 | ρ0HeLa × CM       | Oua + HAT | 12               |

*α, β-resistant.
*b Numbers in parentheses indicate donor ages of fibroblast lines.
*c Seven of 12 clones had more than 95%, two had 87%, and 3 had 35–42% mtDNA with a tRNA Ile 4,269 (A to G) mutation.

d’= resistant.

**Fig. 4.** Recovery from age-related reduction of mitochondrial translation activity specifically observed in fibroblasts from aged subjects by introduction of pure HeLa nuclei. Nuclear hybrid clones NH106 (5, 11, and 12) and NH102 (1, 5, and 8) were isolated by the introduction of pure HeLa nuclei into fibroblast lines TIG106 and TIG102 obtained from 80- and 97-year-old subjects, respectively. Nuclear hybrid clones NH3S (2, 4, and 8) and NHCM (1 and 3) were used as positive and negative controls, respectively; ρ0 He, ρ0 HeLa cells. Upper panel, proteins of the mitochondrial fraction (20 μg lane) separated by SDS-polyacrylamide gel electrophoresis. ND5, COI, ND4, Cytb, ND2, ND1, COII, COIII, ATP6, ND6, ND3, ATP8, and ND4L are polypeptides assigned to mtDNA genes. Lower panel, quantitative estimation of [35S]methionine-labeling of mitochondrial translation products.

**Fig. 5.** Recovery from age-related reduction of COX activity specifically observed in fibroblasts from aged subjects by introduction of pure HeLa nuclei.

Using these nuclear hybrid clones, we compared mitochondrial translation activity by analysis of [35S]methionine incorporation into mitochondrionally synthesized polypeptides. As shown in Fig. 4, all nuclear hybrid clones showed similar mitochondrial translation activities irrespective of whether their mtDNA was derived from fetal or aged subjects, suggesting that all mtDNA-encoded factors necessary for the translation in mitochondria, such as 22 mitochondrial tRNAs and 2 rRNAs, were intact in fibroblasts from the aged subjects. Then, we compared COX activities and found that these phenotypes were also completely restored by the introduction of HeLa nuclei (Fig. 5). Similar results were obtained on comparison of oxygen consumption rate (data not shown). These observations suggest that accumulation of nuclear-recessive mutations of factors involved in mitochondrial translation was responsible for the defects but that all mtDNA-encoded factors necessary for the formation of functional respiration complexes as well as those necessary for the mitochondrial translation were intact. Therefore, mtDNA in the fibroblasts of aged subjects did not contribute to the observed age-related decline of mitochondrial respiratory function in the aged fibroblasts.

Recently, very small amounts of mtDNA molecules with large scale deletion mutations that are not detectable by Southern blot analysis were observed in human (9, 10) and mouse brain (24, 25) by the PCR technique. Since a common mutant mtDNA with a 4,977-bp deletion, ΔmtDNA4977, was found to accumulate preferentially in human brain with increase in age...
used for the last round. Note that R2 was used for the second round, and then the primer set F3 and R3 was employed. In the second round of PCR, the inner primer set F3 and R3 were employed. In the first round of PCR, the outer primer set F1 and R1 (see "Materials and Methods") were employed. In the second round of PCR, the inner primer set F3 and R3 were employed.

For detection of a small amount of a common deletion mutant mtDNA in fibroblast lines, PCR analysis was carried out using total DNA prepared from ρ0 HeLa cells as a negative control (NC) and ΔmtDNA977 prepared from a patient with Kearns-Sayre syndrome (provided from Drs. Y.-i. Goto and I. Nonaka, National Center of Neurology and Psychiatry, Japan) as a positive control (PC). 3S, 2S, 1, 106, 107, and 102 show results for TIG3S, TIG2S, TIG1, TIG106, TIG107, and TIG102, respectively. The amplified products were separated in 2.5% agarose gels containing ethidium bromide (0.1 μg/ml). The fragment of 392 bp is a PCR product amplified from ΔmtDNA977.

In the second round of PCR, the outer primer set F3 and R3 were employed. b, PCR analysis using three sets of primers. For detection of a very small amount of deletion mutant mtDNA, the primer set F2 and R2 was used for second round, and then the primer set F3 and R3 was used for the last round. Note that ρ0 HeLa cells did not show any signals (9, 10), we examined using the PCR technique whether ΔmtDNA977 also accumulated in fibroblast lines from aged subjects. We found that the 392-bp fragment amplified from ΔmtDNA977 was not detectable in DNA samples of all six fibroblast lines from the aged donors and from fetuses (Fig. 6a). On the other hand, when the sensitivity of the amplification conditions was significantly increased, a 279-bp fragment derived from a deletion mutant mtDNA other than ΔmtDNA977 was observed in one fetal fibroblast line (Fig. 6b). Sequence analysis showed that the deletion was 5,090 bp long with a break point from nucleotide positions 8,450 to 13,541, and that the deletion was flanked by a 4-bp direct repeat (5′-AATAT-3′). Thus, even if a very small amount of deletion mutant mtDNA is present in fibroblast lines, it is not associated with aging.

**DISCUSSION**

Since the discovery of the intrinsic limitation for population doubling in cultured human diploid fibroblasts (26), they have been used extensively as models for investigating in vitro cellular aging (27, 28). However, decrease of mitochondrial respiratory function has not been observed in human diploid fibroblasts during in vitro cellular aging, i.e. during increase of their population doubling level of fibroblast cultivation (29) but has been observed during aging in vivo, i.e. with age of the fibroblast donors (11). In fact, increase of the population doubling level did not affect both COX and mitochondrial translation activities in fibroblast lines from fetus and aged subjects (11). This seems consistent with the observation of Goldstein et al. (29) that there was no gross deficit in energy metabolism at increased population doubling level when fibroblasts from the same donor with different population doubling level were compared. The apparent discrepancy between in vivo and in vitro aging could be attributed to the difference of their time scales; in vitro cellular aging lasted only for several months, whereas in vivo aging lasted up to 70–100 years. Therefore, the age-related mitochondrial dysfunction observed in human skin fibroblasts is the phenomenon restricted to in vivo aging.

In this study, we showed that this in vivo aging-related mitochondrial dysfunction could be due at least partly to the reduction of mitochondrial translation activity. We then examined whether similar reduction could be observed in cytoplasmic translation activity in fibroblasts from the aged subjects and found that the age-related reduction was limited to translation in mitochondria (Fig. 3, a and b). Then, to determine which genome, mitochondrial or nuclear genome, is responsible for this age-related, mitochondria-specific defects, pure nuclear transfer was carried out from ρ0 HeLa cells to fibroblast lines from fetal and aged subjects. Results showed that the age-related, mitochondria-specific defects observed in human skin fibroblasts were due to nuclear-recessive mutations of the factors involved in mitochondrial translation, although we could not completely rule out the possibility of contribution of non-nuclear DNA- and non-mtDNA-encoded factors in ρ0 HeLa cells to the correction of the age-related defects.

We previously reported that age-related reduction of COX activity in human skin fibroblasts inherited in a nuclear-recessive way, based on the observation that the reduction was restored by the introduction of pure HeLa nuclei (11). However, in our previous work we used only one nuclear hybrid clone isolated by the fusion of aged fibroblasts with ρ0 HeLa cells and did not examine positive control clones using fetal fibroblasts (11). Therefore, it was possible that we may have picked up by chance a respiration-competent clone or that the apparent restoration of COX activity by the introduction of HeLa nuclei was not sufficient to exclude the involvement of mtDNA somatic mutations in age-related mitochondrial dysfunction. Moreover, we did not prove that respiration-deficient clones could be isolated in selection medium with Oua+ HAT using respiration-deficient fibroblasts as negative controls.

In this study, pure HeLa nuclear transfers to normal fibroblasts from a fetus were carried out as a positive control and to respiration-deficient fibroblasts from a patient with cardiomyopathy as a negative control. The negative control experiment showed that we could isolate nuclear hybrid clones expressing no mitochondrial translation or COX activity (Figs. 4 and 5). Thus, our system for isolation of nuclear hybrids is suitable for isolating respiration-deficient clones expressing no mitochondrial translation or COX activity (Figs. 4 and 5). This method, we showed that the activities of both mitochondrial translation and respiratory function of fibroblasts from all aged subjects were restored to comparable levels to those of fetal fibroblasts by the introduction of pure HeLa nuclei, suggesting that mtDNA in fibroblasts from aged subjects is functionally intact.

Recently, by isolating cybrid clones using a similar mtDNA transfer system to that which we reported previously (11), Laderman et al. (12) reported contradictory observations, suggesting that mtDNA is involved in age-related mitochondrial dysfunction in human fibroblasts. They claimed the occurrence of age-related accumulation of mtDNA mutations in human fibroblasts based on the observations that 5% cybrid clones with mtDNA imported from fibroblasts of elderly subjects showed slightly lower mitochondrial respiratory function than those from younger subjects. However, they did not prove the presence of any mtDNA mutations in the clones with a lower respiratory function. Moreover, since the other 95% clones with mtDNA from elderly subjects showed comparable respiratory...
function to those with mtDNA from younger subjects, their observations could be reinterpreted as showing that most mtDNA molecules in fibroblasts from aged donors do not have more mutations than those from younger subjects. Furthermore, the presence of only 5% clones with a lower respiratory function could hardly explain the age-related shift to 60–80% reduction of the respiratory function in fetal fibroblasts (Fig. 1). Therefore, the apparent discrepancy between the report of Laderman et al. (12) and ours (11) could be due simply to a difference in the interpretation of observations.

In these mtDNA transfer techniques, however, the selection medium without uridine has to be used for isolation of cybrids to exclude parental ρ0 cells (11, 12). This medium could also exclude cybrids expressing respiration deficiency, resulting in the selective isolation of respiratory-competent cybrids. Therefore, in the present work, we did not use mtDNA transfer techniques but used nuclear transfer techniques that do not eliminate clones expressing complete respiration deficiency (Figs. 4 and 5). Then, we compared both mitochondrial translation activity and mitochondrial respiratory function and showed that no respiration-deficient clones were present among 36 nuclear hybrid clones irrespective of whether their mtDNA were derived exclusively from fetal or aged donors (Fig. 5). These observations completely excluded the possibility that accumulation of mtDNA with somatic mutations plays a role in the age-related respiratory defects observed in human skin fibroblasts.

Recently, a deletion mutant ΔmtDNA4977 was found to accumulate preferentially in human brain with increase in age (9, 10). In this study, however, we showed that no ΔmtDNA4977 was detected in mtDNA of any human fibroblast lines from aged or fetal subjects by PCR amplification (Fig. 6). mtDNA4977 was observed in a fibroblast line from a fetus by PCR amplification using three sets of primers (Fig. 6b). These observations suggest that large scale deletion mutant mtDNA molecules, if they do occur, do not accumulate in mitotic cells with age, partly due to selection against the surviving cells containing these deletion mutants (5). On the other hand, they could be propagated in specific conditions, such as in blood cells of patients with Pearson syndrome (30) and in cybrid clones (6). Although the observations of PCR experiments do not exclude the possibility of accumulations of various other unidentified somatic mutations in the mtDNA populations of fibroblasts from aged subjects, our nuclear transfer experiment completely ruled out the possibility of their involvement, at least in age-related respiration defects in fibroblasts.

We recently proposed the idea that mitochondria and the mitochondrial genome function as a single dynamic cellular unit in living human cells by the presence of exchanging mtDNA and its products between mitochondria (31). This was supported from the evidence for the coexistence and cooperation of mutant HeLa mtDNA with chloramphenicol resistance and mutant mtDNA with a large scale deletion originated from organelles of different cells (32). As mtDNA mutations in different genes can complement each other (32), the presence of various kinds of mutant mtDNA molecules in single cells would not have serious additive influence on mitochondrial respiratory function.

All these considerations indicate that age-related accumulation of somatic mutations in mtDNA, even if it occurs in fibroblasts, is not responsible for the age-related decrease in mitochondrial respiratory function observed in human fibroblasts. As the nuclear genome encodes most mitochondrial proteins including factors necessary for expression of the mitochondrial genome (33) and as the defects were at least in part ascribable to reduction of mitochondrial translation activity, we are now investigating nuclear-coded factors particularly involved in mitochondrial translation to understand the precise mechanisms of the age-related mitochondrial dysfunction in human skin fibroblasts.

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