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CORRELATION BETWEEN MITOCHONDRIAL DNA 4977-bp DELETION AND RESPIRATORY CHAIN ENZYME ACTIVITIES IN AGING HUMAN SKELETAL MUSCLES

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SUMMARY. The content of the mitochondrial DNA 4977-bp deletion and the respiratory chain enzyme activities were determined in the same human skeletal muscle specimens. A direct correlation between damage to mtDNA and bioenergetic deficiency was observed. The time-course of the appearance of the mtDNA deletion was followed. The highest percentage of mtDNA-deleted molecules was 0.26% and it was found in the eighties which corresponds to the age of the major reduction in the respiratory chain enzyme activities. Two samples with very low mitochondrial respiratory enzyme activities exhibited much higher levels of deletion compared to the similar age counterparts. Given, however, the low absolute level of the deletion also in these samples, we suggest that damage to the respiratory chain complexes, especially complex IV, might be the cause more than the effect of the increased number of mtDNA molecules bearing deletions in aged human skeletal muscle.

Aging is a very complex process involving changes at morphological and biochemical levels shown by the single cells as well as by the whole organism. Among the different hypotheses about aging the damaging actions of reactive oxygen radicals seem to deserve a special role. A main target of the oxygen radicals should be the mitochondria (1) where 90% of the cell oxygen consumption takes place. Oxygen radical species are, in fact, by-produced in mitochondria during the respiratory chain electron transport accounting for between 1% and 4% of oxygen uptake (2). The oxygen radicals should be rapidly converted into more inert species by the successive actions of SOD, GSH-PX and catalase. However, the lack of a relevant age-related increase of these enzyme activities (3-5) might not counterbalance the increased production of oxygen free radicals described during aging (6, 7). It is, therefore, hypothesizable a more serious damaging action of the oxygen radicals inside the mitochondrion: on membrane lipids, proteins and DNA. Such a multiple damage could explain the age-dependent decrease of the respiration rates and of the activities of different respiratory chain complexes described in several tissues among which in human skeletal muscle (8-11). Particularly affected by age appear to be complex I, complex IV and complex II activities and, since two of them (namely complexes I and IV) include subunits encoded by the mitochondrial DNA (mtDNA), it

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Abbreviations: SOD, superoxid dismutase; GSH-PX, glutathione peroxidase; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

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appeared of particular interest to test the presence and the role of age-dependent accumulated mtDNA mutations. In fact, the mtDNA could be a main target for the mutagenic action of the oxygen radicals because of its close proximity to the major cellular source of such reactive species.

Also the lack of efficient DNA-repair systems and of histone-like proteins to protect the DNA in the organelle make the mt genome copies particularly prompt to mutations. To date different kinds of age-dependent mtDNA mutations have been reported. They include: the level of the 8-hydroxy-deoxyguanosine in rat liver (12) as well as in rat and human heart (13), point mutations in tRNA genes (14, 15) and various sizes deletions in human and rat mtDNA. An increase with age of two human mtDNA deletions, 4977 bp and 7436 bp long, respectively, has been described: in different areas of the cerebral hemispheres (16, 17), in the heart (13, 18), in the liver (19-21) and in the skeletal muscle (9, 22). The 4977 bp deletion, known as the "common deletion" because of its frequency in the mitochondrial encephalomyopathies cases, has been quantitated by means of various methods. It has been suggested that the high percentage of this deletion found in the pathology could prevent mitochondria from a normal respiratory metabolism and from supporting the muscle fibers with the oxidative energy on which they strictly depend, thus causing their progressive degeneration. Since it has been reported that aging could be the most widespread mitochondrial pathology, this deletion has been quantitated also in some tissues of elderly humans. The previous studies about the quantitative dosage of this deleted species (Δ^{4977} mtDNA) in the aging skeletal muscle reported different ranges of accumulation: a 20-fold increase in (9) (from a 21-year-old sample through a 78-year-old one) versus a 10,000-fold increase in (22) (from a 0.5-year-old sample through two 84-year-old samples). However, the respiratory complex enzyme activities and the "common deletion" level have never been measured in the same samples. Therefore, we tried to establish a temptative correlation between the percentage of Δ^{4977} mtDNA and the respiratory chain enzyme activities in a number of samples. We measured complexes I, II, III and IV enzyme activities and performed dosage experiments of the 4977 bp deletion on thirteen subjects of different ages (from 17 to 89 years old). A high increase of Δ^{4977} mtDNA was found around the sixties with a sudden rise of the experimental line approaching then a plateau value from the seventies on. The analysis of the biochemical data of the samples showed a relevant decrease of the enzyme activities from sixty years on, thus indicating a possible correlation with the levels of the Δ^{4977} mtDNA molecules, increasingly produced where the respiratory chain complexes activities appeared less efficient.

MATERIALS AND METHODS

Muscle samples: skeletal muscle biopsies of proximal vastus lateralis (100-200 mg) were obtained, with informed consent, from patients undergoing routine orthopaedic surgery and cryopreserved within 3 hours.

Measurements of the respiratory chain enzyme activities: the assays conditions were as described in (10) and the measurements were performed on isolated frozen-thawed mitochondria.

Preparation of DNA: the skeletal muscle samples were powderized under liquid nitrogen, resuspended in RSB 0.5 ml/100 mg tissue [RSB: 10 mM Tris HCl (pH 7.4), 10 mM NaCl, 25 mM EDTA (pH 7.4)] and digested with 1 µg/µl Proteinase K, 1% SDS at 50°C for 2 hr. 50 µl of 5 M NaCl were added and the DNA was extracted once with an equal volume of phenol, chloroform and isoamyl alcohol (25:24:1) and once with two volumes of diethyl ether. DNA was precipitated with two volumes of ethanol at -80°C for 1 hr (visible nuclear DNA was removed from the sample before storing at -80°C) and then rinsed with 70% ethanol. The precipitated DNA was recovered in deionized sterile water.

Quantitative PCR: The PCR was performed using a Perkin Elmer DNA Thermal Cycler in 100 µl reaction mixtures containing: total DNA (50-1000 ng), 20 µM of each dNTP, 50 pmol of each primer, 2.5 U of Taq DNA polymerase (Boehringher Mannheim, Germany), 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl and 50 µCi of 3000 Ci/mmol α[^{32}P] dATP. The reactions were started by the addition of the enzyme after a 5 min denaturation at 94°C. The PCR profiles were as it follows: 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 25 cycles using the primers ND1-For 5' CCCGATGGTGCAGCCGC 3' (nt 3007-3023 on the L strand) and 3.5 Rev 5' CTAAGGTCGGGGCGGTGAT 3' (nt 3538-3520 on the H strand) for total mtDNA; 1 min at 94°C, 1 min at 59°C and 1 min at 72°C for 30 cycles using the primers Atp-For 5' CCCCTCTAGAGCCCACTGTAAAGC 3' (nt 8282-8305 on the L strand) and 13 Rev-bis 5' CTAGGGTAGAATCCGAGTATGTTG 3' (nt 13928-13905 on the H strand) for deleted mtDNA. The exponential phase of PCR amplification was followed taking, after predeterminate numbers of cycles, reaction aliquots that were run on 5% polyacrylamide minigels (0.75 mm thick, 10 cm wide and 8 cm long) in 1xTBE at 130 Volts (30mA) for 1 hr (Fig. 2A). The radiolabeled PCR products, visualized by autoradiography, were cut out of the gel, dried at 80°C for 4 hr in scintillation vials and counted. The incorporated radioactivity was transformed in concentration (moles/µl) and used in a plot versus the number of cycles. The extrapolation at 0 cycles of the experimental line allowed the determination of the absolute initial concentrations of the template species without need of external amplifications as referring standards.

RESULTS

The results obtained by measuring the respiratory chain complexes enzyme activities in thirteen skeletal muscle samples of different ages are presented in Fig. 1. Before the age of sixty years no major differences in complex I and complex IV activities were revealed with the exception of the 38 and 56 years old samples which exhibited lower values in comparison with similar age controls. After sixty years a relevant reduction of the enzyme activities was observed as a general trend. The presence of Δ^{4977} mtDNA in the above reported thirteen samples was then evaluated. To quantitate this deletion in our thirteen muscle samples the method by (23), already used by us for the dosage of a similar deletion in aging rat mtDNA (24), was chosen. Total mtDNA initial absolute concentration was assessed by following the kinetics of the radioactively-labeled amplification of a rarely deleted region (531 bp long) encompassing part of the 16S rRNA gene, the $tRNA^{Leu}$ gene and part of the ND1 gene (see Materials and Methods for details). The extrapolation to 0 cycle of the

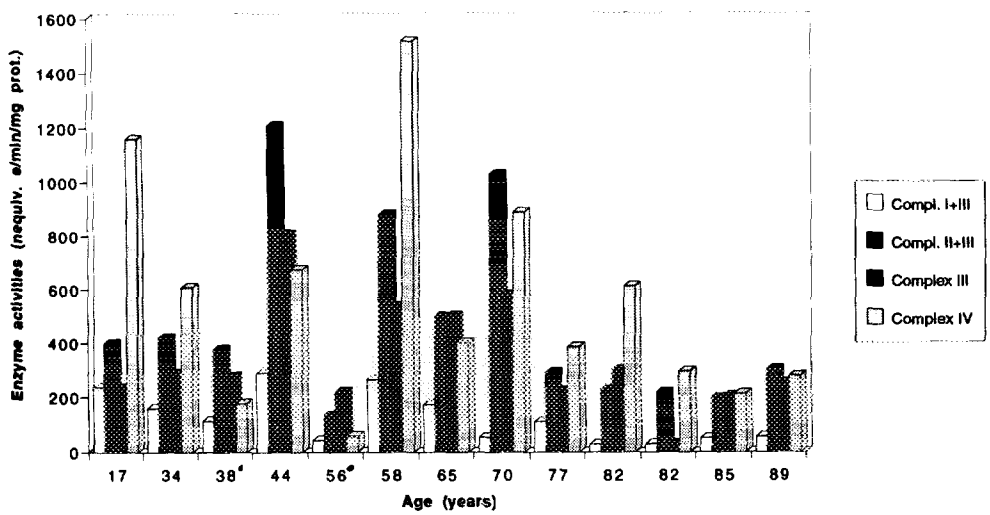

Figure 1. Comparison of skeletal muscle respiratory chain enzyme activities from the samples. The enzyme activities are expressed in nequiv. e/min/mg prot.

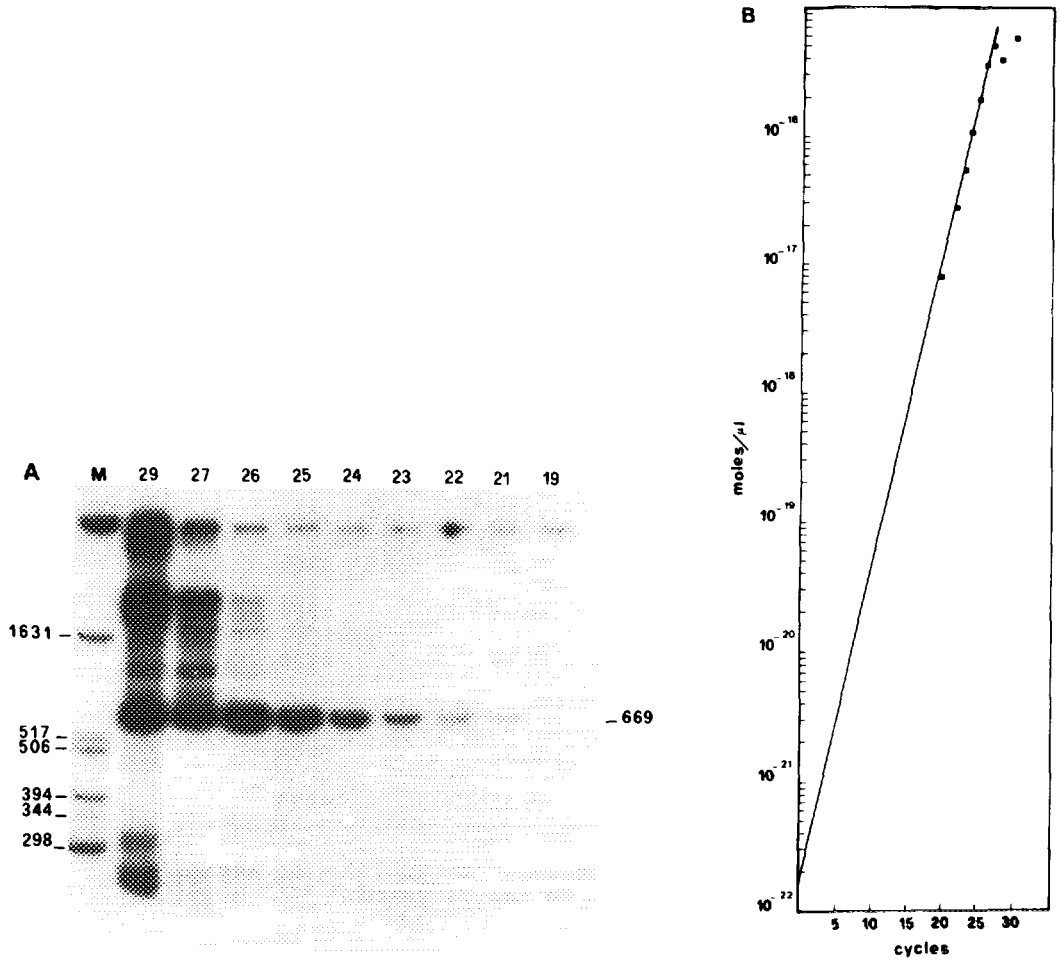

Figure 2. Quantitation of the Δ^{4977} mtDNA template concentration in the 58-year sample.

A. Autoradiogram of the gel showing the progressive increase of the 669 bp product. The numbers on top of the lanes indicate the cycles numbers. M = bp size marker (pBR322 x Hinf I).

B. Semilogarithmic plot of product concentration (moles/µl) vs. cycles number. Dots indicate the experimental values.

straight line representing on semilogarithmic paper the exponential phase of the PCR kinetics permitted the determination of the initial absolute concentration of the total mtDNA template species (including deleted and not-deleted molecules). In an analogous way, the extrapolation to 0 cycle of the PCR kinetics of the deleted mtDNA specific product (669 bp long, see Materials and Methods for details) allowed the determination of the initial absolute concentration of the deleted template species (Fig. 2B). Table 1 reports the Δ^{4977} mtDNA percentage of the samples calculated from the initial concentrations of the two template species, respectively, deleted and total mtDNA. The 4977 bp deletion level increases with age especially around the sixties with the exception of the 38 and 56 years samples, particularly high. The plot in Fig. 3 represents the ratio of deleted mtDNA molecules to total mtDNA ones versus the sample's age. After the sixties the shape of the plot rises to approach a plateau value in spite of the individual samples variability. The highest ratio

Table 1

Age-accumulation of the 4977-bp deletion

Sample age (years)	Del./Tot. %	N. of fold*
17	0	-
34	0.00005	1
38*	0.35*	7000*
44	0.001	20
56*	0.22*	4360*
58	0.07	1320
65	0.06	1300
70	0.22	4400
77	0.26	5200
82	0.26	5200
82	0.2	4000
85	0.26	5200
89	0.21	4200

* = N. of fold increase for Δ^{4977} % of each age relative to the 34 years Δ^{4977} %.

between deleted mtDNA and total mtDNA molecules is reached in the eighties-years samples with the value of 2.6×10^{-3}, that is about 5 deleted molecules for cell assuming a mean of 1,000 mitochondria per cell and a mean of 2 genomes for mitochondrion. Our final value is the highest one since now reported for this deletion, probably because of the higher sensitivity of the used technique. However, our value is about the same order of magnitude of that presented by other Authors (21, 22) and one order of magnitude greater than the value already reported by (9). A special attention has to be paid to two samples: the 38 years old and the 56 years old ones. These individuals show a Δ^{4977} mtDNA percentage value considerably higher than that of other similar age samples (Tab. 1). Comparing these specific samples respiratory chain enzyme activities with the similar age samples values an inversal correlation between respiratory complexes activities and Δ^{4977} mtDNA percentage can be observed (Tab. 2). In fact, the 38 years old and the 56 years old ones present, respectively, a markedly reduced respiratory complex IV activity and respiratory complexes I + III, II + III and IV activities lower than those of the corresponding age samples counterparts (see also 10).

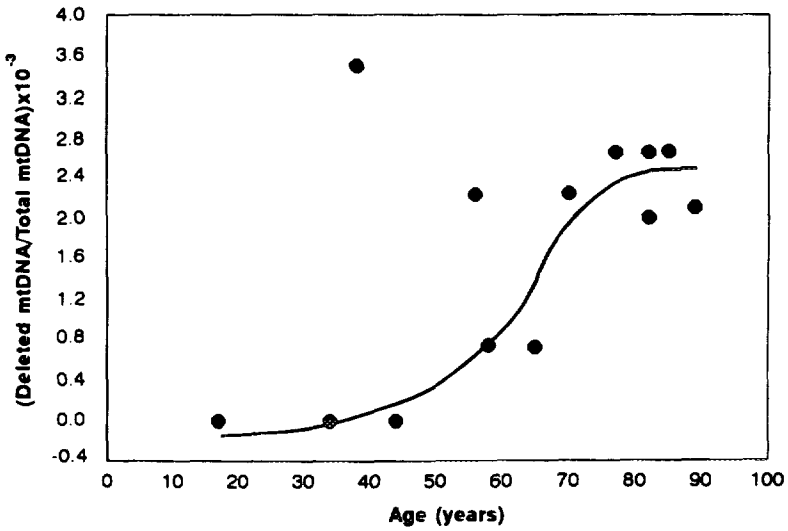

Figure 3. Accumulation of common deletion△mtDNA as a fraction of total mtDNA. Linear plot of△mtDNA fraction vs. age of the sample.

DISCUSSION

The mitochondrial involvement in aging has been clearly demonstrated by a large variety of data: biochemical, histochemical and morphological. Recently the possible role of the mitochondrial nucleic acids in the degenerative phenomena of senescence has been analyzed in several organs, mainly constituted by non-mitotic cells as brain (16, 17), heart (18) and skeletal muscle (9, 11, 21, 22). An age-dependent accumulation of the 4977 bp deletion in mtDNA (Δ^{4977} mtDNA) has been shown in various tissues. In the present study, by means of a very sensitive technique, we have confirmed the age-dependent accumulation of Δ^{4977} mtDNA in skeletal muscle, as it had been shown in other tissues. The highest content (0.26%) of

Table 2

Comparison of respiratory chain enzyme activities and Δ^{4977} mtDNA percentages in similar ages couples of samples

Sample age (years)	34	38*	58	56*
NADH-cyt. c red. (neq. e/min/mg pr.)	160	113	265	43
Succ.-cyt. c red. (neq. e/min/mg pr.)	420	377	878	133
Ubiq.-cyt. c red. (neq. e/min/mg pr.)	289	278	538	219
Cyt. c oxidase (neq. e/min/mg pr.)	609	177	1514	59
Del./Tot. %	0.00005	0.35	0.07	0.22

the deleted species has been found around the eightieth decade (Tab. 1). The plateau (Fig. 3) that seems to be reached at this age could be due to the progressive death of fibers carrying a high concentration of the deletion. The value for the "common deletion" that we measured, in fact, represents the mean level of this deletion in the tissue sample examined since it has been reported that there is not an even distribution of the deletion along the fiber, but a focalized one with different populations of mtDNA molecules in contiguous regions of the same fiber. Besides this, a mosaic-like distribution of fibers becoming progressively Cox-negative with aging has been reported in several muscles (25) increasing the variability due to the single fibers values. The highest value of the "common deletion" percentage in aging subjects never reaches even the lowest value of pathological samples namely the 27% of some mild KSS cases (26). Therefore, questions are still open about the physiological meaning of this and the other mtDNA deletions described in aging samples. It seems that the Δ^{4977} mtDNA percentage increases with age while respiratory complexes functionality decreases as exemplified by the 38 years old subject who presents a respiratory complex IV activity seriously reduced associated to a Δ^{4977} mtDNA percentage much higher than its similar age counterpart (see also 10). Also the 56 years old subject, characterized by a Δ^{4977} mtDNA percentage higher than that of the other fifthieth-class member, shows respiratory complexes IV, I and II activities lower than his counterpart (see also 11). It appears, therefore, possible to establish a relationship between a reduced respiratory chain functionality and an increased mutation level of mtDNA. The age-reduced activity of cytochrome *c* oxidase might enhance the amount of oxygen radicals produced by complex III whose activity does not seem to decrease in aging (10). The consequent increased leakage of oxygen radicals from the respiratory chain (2) might accelerate the mutation rate of mtDNA causing the accumulation of Δ^{4977} mtDNA and the appearance of additional deleted mtDNA species also found in our samples (data not shown and 11). Although it seems that the age-dependent decrease of respiratory chain enzyme activities and increase of the mean level of the "common deletion" in the skeletal muscle are chronologically and quantitatively correlated, the average data on tissues might not allow a thorough understanding of the aging phenomenon. Probably the forecoming *in situ* PCR will permit the determination of the deleted mtDNA content at the single fiber level as well as the real correlation with the respiratory chain enzyme activities. Then it will become clear if the extent of the age-related decrease of some of such activities is the cause or the effect of the accumulation of deleted mtDNA molecules in the largely polyploid mtDNA population in each fiber. This will throw light also on the aspect of the tissue-specific energy tresholds which might be relevant for the shift to an energy-defective situation and its following degenerative phenomena, perhaps involved in the aging process too.

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