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Advanced telomere shortening in respiratory chain disorders

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Cell and tissue damage in respiratory chain disorders have been related to increased production of reactive oxygen species (ROS). We measured telomere lengths in such disorders since ROS have also been implicated with telomere shortening. We investigated whole blood cell DNA of 14 patients with MELAS-related mitochondriopathy and two patients with the LHON-associated G11778A mutation of the mitochondrial genome. The phenotypes were variable and included an unusual case of schizophrenia-like psychosis associated with the A3243G mutation. As compared to healthy controls telomere shortening in the patient group was advanced ($P \leq 0.006$). We compare this finding with the accelerated telomere shortening in Down's syndrome and in chromosomal breakage syndromes. We discuss possible relations between advanced telomere shortening and selective constraints that act on proliferating cells with respiratory chain dysfunction.

INTRODUCTION

Telomeres are specialized structures at the chromosomal ends comprising a simple repetitive $(TTAGGG)_n$-sequence (1). Considering primer dependency and unidirectional activity of DNA-polymerases an 'end replication problem' of linear DNA molecules was postulated (2,3) which should cause incomplete 5′-end synthesis and, thereby, telomere shortening in replicating somatic cells. The detection of telomere shortening *in vitro* and *in vivo* (4–6) justified this conjecture.

However, the end replication problem may not be the only cause of telomere shortening. Increased oxygen tension was shown to accelerate telomere shortening in replicating fibroblasts *in vitro* (7). This acceleration was attributed to an increased number of telomeric single strand breaks leading to the loss of the distal fragments during DNA replication. Indeed, telomeric DNA sequences seem to be particularly prone to chromosomal breakage (8,9), and their GGG-triplets are a major target for reactive oxygen species (10–12).

Increased production of reactive oxygen species has also been related to cell and tissue damage in respiratory chain disorders (13–16). Therefore, we measured telomere lengths in patients with such disorders.

RESULTS

The autoradiograms (Fig. 1, Fig. 2 and Fig. 3) already indicated advanced telomere shortening in the patient group. For statistical analysis we established linear regressions of peak positions versus age.

Statistical analysis

Telomere length variability is considerable and linear regressions may yield r-values of only –0.4 (17). We determined r = –0.65 in the control group and r = –0.46 in the patient group. As compared to the null hypothesis regression was significant in both groups (controls, $12.3 - 0.069 \times$ age, $P \leq 0.002$; patients, $10.0 - 0.054 \times$ age, $P \leq 0.05$). The difference between the slopes was not significant ($P \geq 0.3$). However, the vertical distance (1.9 kb) was highly significant ($P \leq 0.004$). Similar statistical results were found when the two patients with the LHON-associated G11778A mutation (Fig. 3) were neglected (regression, $10.2 - 0.055 \times$ age, r = –0.47, $P \leq 0.05$; vertical distance, 1.7 kb, $P \leq 0.009$).

In the control group the slope was somewhat steeper than in the large group analyzed by Vaziri *et al.* (27) who determined a slope of 0.041. By exclusion of a single, extremely outlying individual (4 years, 17.4 kb) the regression of our control group was corrected to $11.6 - 0.056 \times$ age, r = –0.67, $P \leq 0.0012$, with a vertical distance to the patient group of –1.5 kb, $P \leq 0.006$. For outlier diagnotic we applied both the DFFITS- (size-adjusted cutoff: 0.6) and the DFBETAS-program (size-adjusted cutoff: 0.5) which are included in the SAS software. Both programs identified only one outlier. This was the individual indicated above (DFFITS: 1.2; DFBETAS: 1.2 for intercept, 0.9 for slope).

Similar slopes but significant vertical distance between control and patient group indicated disease-associated phenomena which lose their relative weight with increasing age. Therefore, we established a separate linear regression of the patients not older than 30 years ($n = 10$) which resulted in, $11.1 - 0.120 \times$ age, r = –0.49, $P \leq 0.08$. The steepness of this regression substantiated the hypothesis of early acceleration of the telomere shortening in the patient group ($P \leq 0.19$ for the comparison with the corrected slope of the control group).

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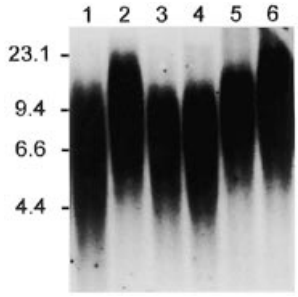

Figure 1. Telomere lengths of a 16 year old MELAS-patient with the A3243G mutation. WBC and muscle DNA of the patient (lanes 4 and 5), WBC and muscle DNA of an age-matched control (lanes 2 and 6), WBC DNA of an 80 year old control (lane 1), WBC DNA of the patient at the age of 14 years (lane 3).

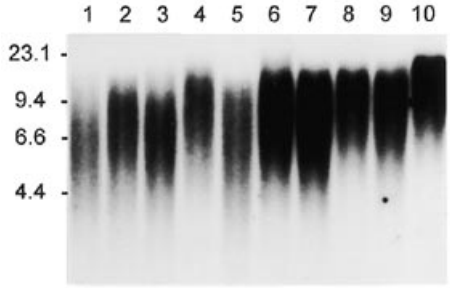

Figure 2. Telomere lengths in WBC DNA of five patients with the A3243G mutation, aged 60 years (lane 1), 38 years (lane 3), 36 years (lane 5), 12 years (lane 7) and 4 years (lane 9) as compared to age-matched controls (lanes i + 1).

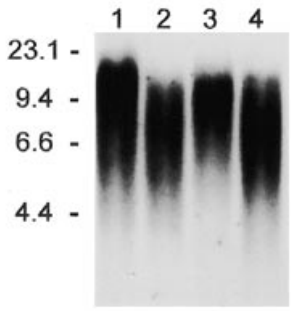

Figure 3. Telomere lengths in WBC DNA of two patients with the G11778A mutation, 25 years (lane 2) and 30 years of age (lane 4) as compared to age matched controls (lanes i – 1).

Telomere lengths in a muscle sample from a MELAS (mitochondrial encephalomyopathy, lactic acidosis, strokelike episodes)-patient with the A3243G mutation were also shorter than in an age-matched control (Fig. 1). This difference may be due to ROS-induced telomere shortening of myoblasts during muscle growth or regeneration.

DISCUSSION

Phenotypes

Spectrum and variability of our patients' clinical signs have been observed before (14,19,20). Nonetheless, the detection of a mitochondrial DNA mutation in a patient with schizophrenia-like psychosis is remarkable. Only one similar case has been reported so far (20) although several findings suggest an association between schizophrenia and mitochondrial dysfunction (18,21,22). Also, the familial risk for schizophrenia is higher in female than in male patients (23–25). This is compatible (18,21) with a mitochondrial mode contributing to the polygenic inheritance component of this disease. A similar disposition in the inheritance of diabetes mellitus (NIDDM) and reports on individual cases prompted recent studies which have shown that 1–2% of all NIDDM cases are related to mitochondrial mutations (26). Therefore, we give notice of our observation although we cannot exclude contingency.

Advanced telomere shortening

We found that the telomeres in leucocytes of patients with LHON (Leber hereditary optic neuropathy)- and MELAS-related mitochondriopathies are, on average, 1.5 kb shorter than those of age-matched controls. Telomeres are favourable targets of oxidative damage due to their multiple GGG-triplets (10–12). Accordingly, increased oxygen tension in cell cultures causes accelerated telomere shortening (7). Thus, our finding is compatible with an increased production of reactive oxygen species (ROS) and must be added to the list of degenerative and dysregulative effects for which ROS have been made responsible in these disorders (13–16).

Abnormal telomere shortening has also been detected in Down's syndrome (27) and in ataxia telangiectasia (AT), a chromosomal breakage syndrome (17,28). In Down's syndrome ROS production is enhanced (29,30). Von Zglinicki *et al.* (7) have assumed that ROS cause telomeric single strand breaks which lead to the loss of the distal fragments during DNA replication and, thus, to accelerated telomere shortening. Indeed, telomeric DNA sequences show increased fragility (8,9). Hence, telomere breakage may be a common cause of telomere shortening in AT, Down's syndrome, and respiratory chain disorders.

The linear regression of telomere length on age which included all of our patients resulted in the same slope, i.e., in the same average rate of loss (–54 bp/year) as the linear regression in the control group (–56 bp/year). However, this does not rule out accelerated telomere shortening in respiratory chain disorders. Indeed, advanced telomere shortening in a patient as compared to an age-matched control indicates that the rate of telomere loss was increased, i.e., that the telomere shortening was accelerated during some earlier period. Yet, accelerated telomere shortening early in life may escape the detection by linear regression analysis that includes patients of all ages: (i) an ascertainment bias is likely to occur due to the extreme variability of the disease. Thus, severe phenotypes (with severe telomere shortening) are likely to be diagnosed early in life while mild phenotypes (with mild telomere shortening) are likely to be diagnosed later. Hence, linear regression of telomere length on age may yield a normal or even subnormal slope. (ii) Southern blot analyses derive average telomere lengths from heterogeneous cell populations. Cells with respiratory chain dysfunction are subject to selective constraints; in the hematopoietic lineage the fraction of mitochondrial genomes with the A3243G mutation declines with increased age (14,31). Assuming that the telomere shortening of the affected cell lines is enhanced, the average shortening is accelerated early in life. However, after some time the affected cell lines will carry

the shortest telomeres of all. Then, the progressive loss of these metabolically deficient cells may actually simulate a normalization or even deceleration of the average shortening. Both explanations (i) and (ii) imply that the quality of the linear regression should be worse in the patient group than in the control group, and that an acceleration of the average telomere shortening could be detectable in young patients. Our results are consistent with these implications (see Results). In case of homoplasmic mutations such as G11778A (14) some acceleration may occur at any age since explanation (ii) is not valid in that case. Subsequent studies will assess the age-associated and subtype-specific dynamics of the advanced telomere shortening that we have described in this paper.

Severe telomere shortening may cause entry into replicative senescence (7,32). Thereby, it might confer a selective constraint itself. Thus, an impact on the development of proliferating cells in respiratory chain disorders must be considered.

MATERIALS AND METHODS

Patients

The patients ranged from 4 to 60 years of age. Thirteen of the 16 patients carried the A3243G point mutation of the mitochondrial genome which is associated with the MELAS syndrome (14). They belonged to four pedigrees. A female patient and her two sons have been described elsewhere (33). Neurosensory hearing loss was the most frequent finding (8 of 13). Diabetes mellitus was found in one, impaired glucose tolerance in two patients. Stroke-like episodes occurred in four patients.

One female patient with the A3243G mutation, 36 years of age, was diagnosed as having schizophrenia at the age of 33. Initially, she presented in a catatonic state which improved upon treatment with haloperidol. Her family reported that a similar episode had occurred two years before. In spite of permanent medication she had to be hospitalized repeatedly. The diagnosis was confirmed by post-episodic exploration when she reported on her impression of being observed and controlled by outside forces, her feeling that thoughts break off or are withdrawn, and on her sensation of acoustic hallucinations. Loose associations and flawed use of words were noticed. Communicative functions were also impaired by flattened affect and emotional ambivalence. Her social behaviour had deteriorated already before diagnosis. CCT scans, neurological exams and neurosensory functions were normal. There was no history of psychiatric disorders among her relatives. Both of her children also carried the mitochondrial mutation.

Two individuals with the A3243G mutation, 12 and 19 years of age, were asymptomatic. One patient, 44 years of age, had the MELAS syndrome but did not show the A3243G mutation. Her diagnosis was confirmed by the detection of complex I deficiency in muscle tissue. Two brothers, 25 and 30 years of age, carried the G11778A point mutation. The older brother presented with spasticity and bilateral visual loss, the younger was asymptomatic.

Point mutations of the mitochondrial genome were detected by allele-specific PCR amplification as described before (33,34). Control samples were taken from 19 healthy individuals, 0–80 years of age. Fifteen controls matched the ages of the 16 patients (±2 years).

Telomere length analysis

DNA samples were digested with *Rsa*I and *Hin*fI (5–10 U/μg; Boehringer-Mannheim, Mannheim, Germany). Complete digestion and absence of unspecific degradation was confirmed by minigel-electrophoresis and ethidium bromide staining of aliquots of each digested sample. Then, 0.5–1 μg of each sample was resolved by 0.7% agarose gel electrophoresis, transferred to a nylon membrane (Hybond-N, Amersham, Buckinghamshire, UK) and hybridized overnight with 5′-labelled $[^{32}P](TTAGGG)_4$ oligonucleotides in 5× SSC at 48°C. Stringency washes were performed in 4× SSC at 48°C. The autoradiography signal was digitized in a Sharp JX-325 scanner using ImageMaster 1D software (Pharmacia Biotech AB, Upsala, Sweden). As others (17) we used the peak of the optical density distribution as the most simple indicator of the mean telomere length. Blackness saturation was avoided by reducing the exposure time of the autoradiograms. The relationship between DNA size and electrophoretic migration was determined by ethidium bromide staining of the DNA molecular weight markers I and II from Boehringer-Mannheim (Mannheim, Germany).

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