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Decreased Number of Mitochondria in Leukoaraiosis

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Background and Aims. Leukoaraiosis (LA), one of the most frequent causes of an age-associated cognitive decline, can be associated with a poor quality of life, leading overall to far-reaching public health problems. Chronic hypoxia of the white matter of the brain may be a factor triggering this entity. LA may develop as a consequence of chronically insufficient cellular energy production and the accumulation of free radicals.

Methods. In this context, after hypothesizing that the number of healthy mitochondria can be crucial in this complex process, a case-control LA study was carried out in which we analyzed the numbers of deleted and non-deleted mitochondria (the common D-loop deletion) per white blood cell. A total of 234 patients with LA and 123 MRI alteration-free subjects served as a control group.

Results. Interestingly, it emerged that the ratio of deleted relative to non-deleted mitochondria is strongly associated with the risk of LA. The calculated K ratio in the LA group was significantly lower than the K ratio in the controls (LA: K 0.37 95% CI 0.05; controls: K 0.48, 95% CI 0.076, $p < 0.001$).

Conclusions. Our study suggests that the ratio of the dmDNA and mDNA can be of great importance in the pathogenesis of LA. © 2015 IMSS. Published by Elsevier Inc.

Key Words: Leukoaraiosis, Mitochondria, Energy production, White matter damage.

Introduction

Leukoaraiosis (LA) is originally a neuroimaging term and refers to hypodensities in CT scans and hyper-intense signals in T2-weighed MRI scans. Although LA is a neuroimaging term, it relates to a complex clinical entity (1−3). LA has been reported to be associated with a cognitive decline and a slowing of mental processing, which can be manifested as a slowness of speech or understanding (4−6). LA is considered to be a vascular demyelinating process (7,8). Although it results from factors such as aging and hypertension (9), development of LA may be associated with a number of several genetic susceptibility factors (10−15).

These genetic and other risk factors suggest that chronic hypoxia might trigger intracellular biochemical events, which result in LA (16,17). Accordingly, we hypothesized that a key role in the demyelinization process of LA may be played by chronically insufficient energy production (18). Hence, in addition to certain biochemical features, the number of mitochondria, the main energy-supplying organelles in mammalian cells (19), may be deterministic for the fate of neurons and glia cells. Mitochondrial mutations can affect different cell types in different ways; this is reflected in mitochondria-associated genetic disorders such as MELAS (20). In such cases, mutations of the mitochondrial DNA or tRNA bring about a malfunction in the affected mitochondria (21). However, virtually no data are available as concerns the number of the mitochondria

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themselves, which must be an important feature from an energetic aspect.

Although the numbers of mitochondria are probably essential as concerns the neurons and glia cells (17), these data cannot easily be examined in clinical studies. However, the numbers of mitochondria in other tissues, which are functionally in constant contact with the brain tissue, may be informative. The white blood cells, for example, constantly circulate and communicate with the brain tissue. Their energy store may be important for the maintenance of the brain because they act as free radical scavengers and also check on the integrity of the neurovascular system of the brain (22). The white blood cells, vascular cells and brain tissue most likely behave as a functional unit. However, very few data have been published as regards the numbers of mitochondria among the different cell types. The number of mitochondria in the white blood cells may be an indicator of the overall number of mitochondria in the given individual.

In this context, we examined whether there is an association between the number of mitochondria in the white blood cells and the development of LA. Besides the genetic heterogeneity of the mitochondrial DNA (23), there are two large types of mitochondrial DNA: one with D-loop sequences and one without them. The former is known as mitochondrial DNA (mDNA) and the latter as mitochondrial DNA with a large and common deletion of 123-1256 bp (dmDNA). This latter DNA might be considered to malfunction in some way (24,25). In our present study we identified the copy number per white blood cell for both mDNA and dmDNA.

Patients and Methods

Study Population

A total of 234 patients with LA were recruited after a clinical scrutiny. LA was diagnosed on the basis of MRI scans as described previously (14). Patients with degrees of I or II on the validated Fazekas LA scale (26) were excluded as were patients with clinical entities such as multiple sclerosis, trauma, postinfectious demyelination, radiation therapy, chemotherapy and well-defined hereditary leukoencephalopathy because these did not result from vascular and chronic hypoxia-triggered demyelination. The patient group consisted of 234 subjects with LA defined as irregular periventricular hyperintensities extending into the deep white matter in T2-weighted MRI scans (grade 3 periventricular hyperintensities) and deep white matter hyperintensive signals with beginning confluence of the foci or with large confluent areas in the T2-weighed MRI scans (deep white matter hyperintensive grade 2−3 signals).

One hundred twenty three MRI alteration-free subjects with no neurological complaints recruited from a population pool in our regional register served as a control group. The clinical risk factors, defined as described in an earlier leukoaraiosis study (27), and basic psychological data were registered in the controls.

DNA Isolation

Genomic DNA was extracted from 200 μl of peripheral blood anticoagulated with EDTA. The leukocyte DNA was isolated by a desalting method (28) and was stored at −20°C until further use.

Determination of Absolute Number of Mitochondria per Leukocyte

The TaqMan real-time PCR assays, specific for the amplification of mDNA and dmDNA, were established by using a specific set of primers and probes. Quantitative mDNA and dmDNA amplification data were normalized to GAPDH as an internal reference gene, which was co-amplified simultaneously in a single-tube assay. The primers were obtained from Sigma-Aldrich (St. Louis, MO) and the sequences are listed in Table 1. Thermal cycling was performed on the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA). Amplification reactions (10 μl each) were performed in duplicate with 1 μl of template DNA. The amplification mix contained the following ingredients: 5 μl of iQ Multiplex Powermix (Bio-Rad), 0.4 μl of each primer, 0.1 μl of each fluorogenic probe, and 1 μl of sample DNA in a total volume of 10 μl

Table 1. Sequences and concentrations of primers and probes

Primer	Sequence	TaqMan signal dyes	Conc. (mmol)
GAPDH for	5′-TCC AGT ATG ATT CCA CCC ATG GCA-3′	-	0.4
GAPDH rev	5′-TTC TAC ATG GTG GTG AAG ACG CCA-3′	-	0.4
GAPDH probe	5′-TTC CAT GGC ACC ATC AAG GCT GAG AA-3′	ROX/BHQ2	0.2
mtDNA for	5′-ATG GCC AAC CTC CTA CTC CTC ATT-3′	-	0.4
mtDNA rev	5′-TTA TGG CGT CAG CGA AGG GTT GTA-3′	-	0.4
mtDNA probe	5′-CGC AAT GGC ATT CCT AAT GCT TAC CG-3′	HEX/TAMRA	0.1
dmtDNA for	5′-ACA CAA ACT ACC ACC TTT GGC AGC-3′	-	0.4
dmtDNA rev	5′-TTC GAG TGC TAT AGG CGC TTG TCA-3′	-	0.4
dmtDNA probe	5′-CCA AAG ACC ACA TCA TCG AAA CCG CA-3′	FAM/TAMRA	0.1

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Table 2. Characteristics of patients and control subjects

Characteristics	Leukoaraiosis $n = 234$	Controls $n = 123$
Sex (females, males)	123, 111	65, 58
Age, years	71.6 ± 10.8[a]	59.4 ± 8.62
BMI, kg/m^2	24.6 ± 2.33	24.4 ± 3.42
Cholesterol, mmol	5.91 ± 1.24	5.52 ± 1.56
Triglycerides, mmol	1.48 ± 0.91	1.50 ± 0.82
Hypertension	46.6%[a]	22.0%
Diabetes mellitus	17.9%[a]	4.88%
Smokers	29.9%	28.5%
Drinkers	12.0%	9.0%

[a] $p < 0.05$; the leukoaraiosis group was compared with the control group by means of the χ^2 test or Mann-Whitney test.

per single-tube reaction. The assay conditions were 3 min at 95°C and 44 cycles of 95°C for 10 sec and 60°C for 45 sec. Quantification was evaluated by the comparative CT (threshold cycle) method (29).

Because no data were available concerning the stability of the number of mitochondria in white blood cells, the numbers of mitochondria in five patients and five controls were determined three times with a minimum interval of 1 month between blood drawings in the same subject. We found merely minimal number differences between the different blood samples in the same subject (data not shown).

Statistical Analysis

Clinical variables in the LA and control groups are listed and compared statistically in Table 2.

mDNA and dmDNA contents per white blood cell in the LA group were compared with those in the control group by means of the two-paired *T*-test (Table 3). In order to make the comparison biologically more sensitive, the mathematical difference between the mDNA and mdDNA contents per cell were derived by the sum of mDNA and dmDNA contents per cell in the same individual. This calculated ratio (K) indicated the weight of uncompensated dmDNA per cell:

$$K = \frac{(\text{mDNA content} - \text{dmDNA content})}{(\text{mDNA content} + \text{dmDNA content})}.$$

K values for each of the LA patients and the controls were compared statistically between the LA and control groups by means of the two-paired *T*-test (Table 3).

Univariate statistical analysis was followed by a logistic regression comparison involving the age, hypertension and diabetes mellitus (due to the statistical difference between the LA and control groups) in addition to the calculated values of K.

Table 3. Distributions of deleted and non-deleted mitochondrial DNA contents per cell in the leukoaraiosis and control groups

Genotypes	Leukoaraiosis $n = 234$ (mean ± 2 SD)	Controls $n = 123$
Number of non-deleted mitochondria per cell	5.5 ± 0.45	5.4 ± 0.5
Number of deleted mitochondria per cell	3.45 ± 0.5	3.5 ± 0.5
K[a]	0.37 ± 0.03	0.48 ± 0.03[b]

[a] Non-deleted mitochondrial DNA content per cell minus deleted mitochondrial DNA content per cell divided by the sum of the contents of deleted and non-deleted mitochondrial DNA per cell (K = [mDNA − dmDNA]/[mDNA + dmDNA]).
[b] $p < 0.001$: the leukoaraiosis group was compared with the control group by means of the two-paired *t*-test.

Results

The frequency of hypertension in the LA group was higher than that in the controls (Table 2). mDNA and dmDNA content did not differ statistically between the LA and control groups (Table 3).

The calculated value of K for the LA group was significantly lower than that for the controls after the used logistic regression analysis. This logistic regression analysis was carried out for the differences between the LA and controls with regards to the age, hypertension and diabetes mellitus. In this way, we could diminish the confounding effects of these clinical factors because they could also be associated with the reduced number of mitochondria (LA: K 0.37, 95% CI 0.05; controls: K 0.48, 95% CI 0.076; $p < 0.001$).

Discussion

Although the basic mDNA and dmDNA contents were statistically the same in the LA and control groups, the value of K was significantly lower for the LA group than that for the control group. This was a mathematical indicator of a larger proportion of dmDNA. dmDNA can potentially result in a mitochondrial malfunction in the following ways: (a) lower energy production; (b) a lower free radical scavenging capacity; (c) a lower rate of adaptation to the prevailing demand for energy production; (d) a narrower range in the adjustment to the prevailing energy demand; (e) a lower of metabolic function capacity in general; (f) a larger extent of free radical production; and (g) a general malfunction of the mitochondrial genetic regulation.

No genetic and biochemical data are available to suggest which of these postulated mechanisms actually exist, but a lower and narrower energy capacity appears probable as the main pathomechanism behind LA. It was demonstrated earlier at a molecular level that LA can result from a very slight, but chronic, level of hypoxia, which can be caused by various environmental and genetic susceptibility factors

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(16). Our present findings are in accord with the earlier findings that uncoupling protein genetic variants play roles in the development of LA (30). The uncoupling proteins govern the electrochemical gradient between the inner and outer spaces of the mitochondria (31), this gradient being essential for the energy production of the mitochondria. If dmDNA is associated with any kind of biochemical malfunction, an uncompensated and larger proportion of dmDNA in the cells can be unfavorable from an energetic aspect.

Our results revealed that the lower the difference between the contents of mDNA (which compensates malfunctions of the dmDNA) and dmDNA the larger the risk of LA in the given individual.

In conclusion, our study suggests that the ratio of dmDNA and mDNA contents can be of great importance in the pathogenesis of LA. These results indicate the need for new approaches for examination of mitochondrial contents in other common brain disorders.

Study Limitations

The numbers of mitochondria in the affected brain tissues could not be examined because this was a clinical study carried out in a human patient population. Brain biopsies would not have been ethical; thus, it remained to identify associations between the numbers of mitochondria in different human tissues. Although we found no apparent change in the number of mitochondria in a small cohort of study subjects during several weeks (which involved several turnovers of the mitochondria in the white blood cells), insufficient scientific data are available concerning the stability of the numbers of mitochondria in the different tissues. This should be clarified.

No examinations were carried out in this study to identify the features of the malfunctioning of the mitochondria with deletion DNAs. However, these limitations do not greatly affect the present results because they are not obviously associated with the findings. Moreover, these postulated limitations are rather scientific questions, which should be considered in subsequent examinations.

Albeit the logistic regression statistical method has greatly decreased the confounding effects of the clinical factors such as age, hypertension (32,33) and diabetes mellitus (34), the results need to be confirmed by a larger population study.

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