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Changes of mitochondria in atherosclerosis: Possible determinant in the pathogenesis of the disease

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ABSTRACT

Electron-microscopic analysis of atherosclerotic lesions demonstrated a high variability in the ultrastructural appearance of mitochondria in human aortic atherosclerotic lesions compared with the appearance of mitochondria in the normal parts of the aortic intima. This prompted us to suggest that the structural variations in the appearance of mitochondria might reflect the existence of somatic mutations in the human mitochondrial genome which could be a determinant of atherosclerosis. To test this hypothesis, we have compared the levels of heteroplasmy for several mitochondrial mutations previously proposed to be associated with different types of atherosclerotic lesions. The homogenates of unaffected aortic intimae and lipofibrous plaques of 12 male aortas were compared to reveal the average level of heteroplasmy for A1555G, C3256T, T3336T, G12315A, G14459A, and G15059A mutations of human mitochondrial genome. It has been shown at least four mutations of mitochondrial genome, namely, A1555G in MT-RNR1 gene, C3256T in MT-TL1 gene, G12315A in MT-TL2 gene, and G15059A in MT-CYB gene have significantly higher prevalence and mean value in lipofibrous plaques as compared to non-atherosclerotic intima, and therefore are associated with atherosclerosis. Somatic mutations in the human mitochondrial genome might play a role in the development of atherosclerosis. The mitochondrial mutations observed in our study should encourage further exploration of the concept that mitochondrial DNA heteroplasmy might be used as a biomarker of atherogenesis.

1. Introduction

Many hypotheses to explain atherosclerosis have been developed, including those involving cholesterol deposition in arterial wall, viral or bacterial infection, response-to-injury, local arterial inflammation and autoimmune response. Although the existing hypotheses are not mutually exclusive, there is no "universal" theory which would explain every clinical observation and would predict how we can defeat atherosclerosis.

In search of the features of atherosclerosis which have not received much attention so far we analysed the ultrastructural characteristics of intimal cells in human atherosclerotic lesions and noted that there is a high variability in the ultrastructural appearance of mitochondria in human aortic atherosclerotic lesions compared with the appearance of mitochondria in the normal parts of the aortic intima (see Results section). This observation prompted us to consider a possibility that the structural variations in the appearance of mitochondria might reflect the existence of somatic mutations in the human mitochondrial genome which could be a determinant of atherosclerosis.

Mitochondria are referred to as semi-autonomous because, unlike any other organelle, they have their own DNA (mtDNA) that codes for the production of four of the five enzyme complexes critical for oxidative phosphorylation. The human mitochondrial genome is comparatively small consisting of only 16,569 base pairs. The mitochondrial genome encodes for 13 proteins involved with oxidative phosphorylation as well as 22 tRNAs and 2 rRNAs involved in synthesis of these mitochondrial complexes. Each mitochondrion contains 2–10 copies of the circular, supercoiled,

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double-stranded DNA found unprotected within the inner mitochondrial membrane. This circular DNA is attached, at least transiently, to the inner mitochondrial membrane. Mitochondrial DNA is particularly susceptible to reactive oxygen species generated by the respiratory chain due to their close proximity. Though mtDNA is packaged by proteins and harbours significant DNA repair capacity, these protective functions are less robust than those functions operating on nuclear DNA and, therefore, are thought to contribute to the enhanced susceptibility of mtDNA to oxidative damage, resulting in high occurrence of mutations. It should be noted that the penetrance and expression of mitochondrial mutations vary greatly and depend mainly on a genotype and the level of heteroplasmy (a mixture of mutant and normal molecules of DNA).

Mutations of mitochondrial genome may result in defects in the protein chains of respiratory enzymes and tRNAs that are synthesized in mitochondria, therefore producing oxidative stress and increasing the probability of atherosclerotic plaque formation. Therefore, studying associations between mitochondrial mutations and focal development of atherosclerotic lesions in the intimal layer of human arteries is of high theoretical and practical impact. It is reasonable to study the physical association of mitochondrial mutations with atherosclerotic lesions, the aspect still remaining obscure. Recently we have suggested that mutations within the mitochondrial genome may be a probable cause of atherosclerosis development in humans [1]. Since a qualitative and a quantitative evaluation of a mutant allele of mitochondrial genome are both necessary for studying the association of mitochondrial mutations with human diseases, we have proposed a method of direct quantitative assay of mutant alleles in the mitochondrial genome based on pyrosequencing of short PCR-fragments of DNA, designed for the measurement of heteroplasmy level in tissues [2]. Using this methodological approach, we have demonstrated that atherosclerotic intimal homogenates differed from unaffected tissue with the respect of the mean level of heteroplasmy for several mutations of mitochondrial genome [3].

In the present work the morphological observation of high variability in the structural appearance of mitochondria in atherosclerotic lesions prompted us to undertake a study, in which we have focused on the difference between unaffected human aortic intima and lipofibrous plaques.

2. Material and methods

2.1. Tissue specimens and procedures for selection of intimal areas for further analyses

Thoracic aorta samples were collected at autopsy 1.5–3 h after sudden death from 12 males aged between 52 and 68 years. The study was carried out in accordance with the principles outlined in the Helsinki Declaration of 1975, as revised in 1983. The study was approved by the institutional review committee of the Institute for Atherosclerosis Research, Skolkovo Innovation Center, Moscow, Russia.

The arteries were opened longitudinally and washed with phosphate-buffered saline (PBS), pH 7.6. The grossly normal parts of the arteries and those regions with atherosclerotic lesions were identified macroscopically and classified according to the classification of the Atherosclerosis Council of the American Heart Association [4,5], utilizing the corresponding histological evaluations. The presence of both normal (unaffected) areas and lipofibrous plaques within the same autopsy sample was the criterion for selection of given sample for the study. Unaffected areas were defined as tissue samples with smooth luminal surfaces, without extracellular lipid droplets in the connective tissue matrix. Lipofibrous plaques were defined as spherical or elliptic protrusions of yellowish or nacreous colour. Microscopically, they included accumulated intracellular lipids and increased amounts of extracellular matrix. Lipofibrous plaques contained a bulky necrotic core covered by a connective tissue layer and also included adjacent zones that morphologically resembled fatty streaks.

2.2. Electron microcopy

The study was designed in order to compare the structural characteristics of mitochondria from lipofibrous atherosclerotic lesions with those in the normal aortic wall. For further electron microscopic examination, tissue samples were fixed in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, at 4 °C, postfixed in 1% osmium tetroxide and then were routinely processed and embedded in araldite resin blocks. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Hitachi H700 electron microscope.

2.3. Genetic analysis

Lipofibrous plaques and grossly normal (unaffected) tissue samples taken from the same aortas were selected for analysis. All 12 autopsy samples contained unaffected (non-atherosclerotic) zones which were estimated to constitute 10%–35% of the luminal surface. All samples had zones with initial lesions and fatty streaks, which were not taken for analysis in this study; the ratio between surface areas of these zones varied greatly. Lipofibrous plaques occupied from 10% to 25% of luminal surface in these samples. Fibrous plaques were present only in 2 aortic samples and occupied from 3% to 10% of the luminal surface. Such a pattern of the distribution of atherosclerotic lesions throughout the luminal surface made it impossible to carry out an analysis of relation of heteroplasmy levels to the severity of atherosclerosis, due to the low statistical power.

Intima was separated from media mechanically by forceps and frozen in liquid nitrogen. All histologically verified segments of intimae from lipofibrous plaques or unaffected regions were combined and homogenized with Potter-Elvehjem Safe-Grind tissue grinder (Wheaton Science Products, USA). After careful stirring, DNA samples were obtained using commercially available kits for DNA extraction (BioRad, England).

For the amplification of fragments of mitochondrial DNA by polymerase chain reaction (PCR) method was used followed by pyrosequencing. Based on the results of previous studies [2,3], mtDNA mutations A1555G, C3256T, T3336C, G12315A, G14459A, and G15059A were selected for comparisons of heteroplasmy levels between unaffected intima and lipofibrous plaques. The nucleotide sequences for forward primers, reverse primers, and sequence primers are described in Table 1. The buffer solution for PCR contained 2.5 mM $MgCl_2$ for A1555G, C3256T, T3336C, and G12315A mutations, and 1.5 mM $MgCl_2$ for G14459A and G15059A mutations. Annealing phase was held at 50 °C for C3256T, T3336C and G15059A mutations, and 55 °C for A1555G, G12315A and G14459A mutations. Extension phase of PCR was held at 72 °C. To quantitatively evaluate mutant alleles, a method of pyrosequencing [6–8] was adapted for conditions where both normal and mutant alleles were present in a biological specimen [2]. Briefly, the defective allele was quantified by analysing the peak heights in the pyrogram of one-chained PCR-fragments of a mitochondrial genome. The percent of heteroplasmy was calculated for each mutation, taking into account the expected sequence and the dimension of peaks for the homozygotes possessing either 100% of the normal or 100% of the mutant allele, as described previously [2].