Mitochondrial DNA point mutation in human oocytes is associated with maternal age

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Abstract

Mitochondrial DNA (mtDNA) point mutations are known to accumulate in an age-dependent fashion in somatic tissues. This study investigated whether a point mutation (T414G) in the mtDNA control region was present in oocytes from women of advanced age. In all, 66 non-viable discarded human oocytes were analysed for the presence of a T414G transversion mutation. DNA sequence analysis confirmed the presence of this mutation in one oocyte from 11 patients between the ages of 26 and 36 years (n = 23), compared to 17 oocytes from 10 patients between the ages of 37 and 42 years (n = 43). The younger group exhibited this mtDNA point mutation in only 4.4% of oocytes compared to 39.5% from the older group (P < 0.01). Therefore, single human oocytes contain the mtDNA T414G transversion point mutation that accumulates in an age-dependent manner. The potential significance of this point mutation may be its association with reproductive senescence. Furthermore, since this mutation exists in the control region of the mtDNA it may affect the regulation of mtDNA transcription and replication during oocyte and post-embryonic development.

Keywords: age-dependent, human oocyte, mitochondria, mitochondrial DNA point mutation, reproductive senescence

Introduction

Mitochondria contain specific DNA (mtDNA) that is distinct from nuclear DNA. The human mitochondrial genome is 16 569 base pairs (bp) of circular double-stranded DNA encoding 22 tRNA, two rRNA, and 13 mitochondrial proteins (Anderson et al., 1981). Although the mitochondria provide some proteins for the oxidative phosphorylation (OXPHOS) pathway, nuclear DNA encodes the majority of proteins and these proteins are imported into the mitochondria from the cytoplasm.

Deleterious mtDNA rearrangements cause cellular energy deficiencies and lead to degenerative diseases affecting the brain, heart, skeletal muscle, kidney, bone marrow, and pancreatic cells. mtDNA rearrangements accumulate with age even faster in non-dividing tissues such as muscle and brain (Cortopassi and Arnheim, 1990; Hattori et al., 1991). When ageing tissues accumulate rearrangements the percentage may reach a significant level and thus a reduction in OXPHOS efficiency can occur (Richter et al., 1988; Hattori et al., 1991). It has been postulated that oocytes, which are non-dividing cells, may also accumulate mtDNA rearrangements, although the relationship with reproductive senescence is still ambiguous (Keefe et al., 1995; Chen et al., 1995; Brenner et al., 1998; Barritt et al., 1999).

Several laboratories have reported the presence of various mtDNA rearrangements in human oocytes (Keefe et al., 1995; Chen et al., 1995; Brenner et al., 1998; Barritt et al., 1999). The most common rearrangement in somatic tissue is a 4977 bp deletion present in patients associated with Kearns-Sayre syndrome and chronic progressive external ophthalmoplegia.
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(CPEO). Even though this mutation was found to accumulate in an age-dependent fashion in many somatic tissues, there was no association found with its presence in the human oocyte, suggesting that this deletion may not be a marker for reproductive senescence (Brenner et al., 1998; Barritt et al., 1999). Twenty-three novel rearrangements with deletions, insertions and duplications have also been found in human oocytes (Barritt et al., 1999). However, there was no significant age-related increase in the number of oocytes containing any of these mtDNA rearrangements.

Presently our laboratory has been investigating whether the frequency of mitochondrial mutations in the oocyte increases with advancing maternal age and thus affects mitochondrial function. These experiments have been confounded by the fact that there is a considerable degree of unexplained variability in the number of mitochondrial genomes in human oocytes (Steuerwald et al., 2000). The number of mitochondrial genomes present in mature human oocytes has been estimated by fluorescent rapid cycle DNA amplification, which is a highly sensitive technique to quantify mtDNA copy numbers in individual cells. Those results show 90 000 to 1 000 000 copies of mtDNA in each human oocyte. Particularly interesting is the possibility that the degree of variation may increase with advancing maternal age (Steuerwald et al., 2000; J Barritt, unpublished results). The relationship between copy number and function remains to be investigated. However, a minimum amount of energy or ATP production is required for normal development of mammalian oocytes and preimplantation embryos (Van Blerkom et al., 1995).

So far, the only point mutation that has been examined in human oocytes is the mtDNA T8993G mutation associated with Leigh syndrome. Individual oocytes retrieved from a patient afflicted with this disease either contained a very high or an undetectable level of mutant mtDNA (Blok et al., 1997). Recently, the presence of a new high copy number mtDNA point mutation in the replication control region has generated considerable excitement in the field (Michikawa et al., 1999). Most strikingly, this thymine (T) to guanine (G) transversion mutation at bp 414 appears in fibroblasts from normal individuals >40 years of age. The presence of this newly discovered somatic tissue point mutation, in the control region, may affect the replication of the mitochondrial genome. Although age affects every aspect of the female reproduction system, the oocyte is believed to be the major target. Several lines of evidence suggest that human oocytes may harbour mitochondrial point mutations (Blok et al., 1997; Brenner et al., 1998; Barritt et al., 1999). This study was undertaken to examine whether there was an age-dependent point mutation in the mtDNA control region of human oocytes.

Materials and methods

Internal review board and patient selection

This investigation was performed according to the guidelines set by a protocol approved by the Internal Review Board (IRB) of Saint Barnabas Medical Center using discarded non-viable human oocytes in the IVF clinic. In this laboratory oocytes that are immature at intracytoplasmic sperm injection (ICSI) or fail to fertilize 20-24 h after insemination or ICSI are considered to be non-viable. Patients were counselled and consented to using non-viable oocytes from their assisted reproductive cycles.

Tissue collection and DNA isolation

Non-viable oocytes 20-24 h after insemination or ICSI were used in this study (GV (n = 2), MI (n = 6) and MII (n = 58)). They were collected and washed individually in phosphate buffered saline after removal of the zona pellucida, cumulus and any remaining spermatozoa by acidified Tyrode’s solution. Samples were frozen at −70°C until DNA isolation and polymerase chain reaction (PCR) amplification. Oocyte DNA was isolated by adding 5 μl of GeneReleaser (Bioventures Inc., Murfreesboro, TN, USA) and microwaving the samples for 5 min at 750 watts.

Mitochondrial DNA amplification

The mitochondrial genome was amplified from non-viable oocytes using PCR. The ~200 bp product was amplified from the above tissues using the primers DP6-3 (5'-TGGCCACACGACCTTAAACAC-3') and DP6-4 (5'-GTGTGTGTGTTGGGTAGATGG-3') using PCR conditions for a 50 μl reaction including 100μmol/l of each dNTP, 5 μl 10X PCR buffer with 1.5 mmol/l MgCl2, 27.5 μl sterile water, 2 units of AmpliTaq DNA Polymerase, 25 pmol of each primer, and 10 μl of 5X Q-solution (Qiagen, Sacramento, CA, USA). The reactions were carried out in a Perkin-Elmer 9600 thermocycler as follows: 5 min at 80°C, 96°C for 3 min; 45 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 1 min; and a final extension of 7 min at 72°C. PCR reactions were hot-started by adding the Q-solution and the primers after the 5-minute inactivation of the GeneReleaser at 80°C. A 10 μl aliquot of the reaction product was run on a 2% agarose gel pre-stained with ethidium bromide, and photographed under UV illumination.

Sequence analysis

The PCR reaction products were purified according to the manufacturer’s directions in the QiaQuick PCR purification kit (Qiagen) prior to DNA sequencing. Sequencing was performed on the purified PCR fragments using an ABI 377 automated DNA sequencer (Retrogen, San Diego, CA, USA). The DNA chromatographs were analysed for the presence of both a T and G nucleotide at base pair position 414 of the mtDNA control region of each individual human oocyte.

Statistical analysis

The data were analysed by both χ² analysis and Student’s t-test for any relationships with maternal age or pregnancy outcome. Patients older than 37 years of age were considered to have diminished ovarian reserve and therefore were classified as “advanced maternal age” in the IVF clinic.
Results

A total of 66 cells was analysed by DNA sequence analysis to confirm the presence of the mtDNA point mutation T414G. Although not precisely quantifiable, the T414G mutation could be detected, particularly when abundant, by direct DNA sequencing of the PCR product. In addition, the presence of a G nucleotide at base pair 414 was confirmed by sequence analysis of the opposite mtDNA strand to confirm the presence of this mutation. An example is shown in Figure 1. Oocytes (n = 23) from 11 patients between the ages of 26 and 36 years, and oocytes (n = 43) from 10 patients between the ages of 37 and 42 years were analysed for the presence of this mtDNA mutation. Only one oocyte (1/23; 4.4%) from the younger age group exhibited this mutation, whereas 17 oocytes (17/43; 39.5%) from the older age patients exhibited this mutation.

This demonstrates a highly significant difference by $\chi^2$ analysis ($P < 0.01$) between the two populations (Figure 2). The number of oocytes/patient (as near as possible to 1–2) and the number of patients in this study did not allow for meaningful cohort analysis. The average age of the patients harbouring this point mutation was 37.7±2.4 years, while the average age without the mutation was 34.7±5.0 years. This was a statistically significant difference using Student’s $t$-test ($P = 0.0385$). Pregnancy and implantation outcomes of the cohort embryos (transferred to the clinical patient) did not relate to the incidence of the mutation. Two of the six patients whose oocytes contained the point mutation had a fetal heart beat (FHB), while 10 of the 15 patients without the mutation in their oocytes had a FHB (not significant). No statistical differences were found based on insemination type or the maturational stage (GV, MI of MII) of the oocytes.

Discussion

Progressive damage to mtDNA during life is thought to contribute to ageing in somatic tissues. However the key link between mitochondrial mutations and ageing has been difficult to reconcile since it is unclear what frequency of the somatic mutation is necessary to alter mitochondrial function. This has raised some fundamental questions about the functional significance of mtDNA mutational damage. Recently the examination of human fibroblasts from older individuals revealed a high copy number mtDNA point mutation in the replication control region (Michikawa et al., 1999). This new mutation, a T414G transversion, was most strikingly found in a high proportion (57%) of older individuals but absent (0%) in all younger individuals.

Whether or not there are age-related mutations or rearrangements in the mtDNA of human oocytes still remains a fundamental question in the reproductive field. In this study, 66 oocytes were analysed by direct DNA sequence analysis for the presence of the T414G mutation. Although not precisely quantifiable, this method reliably detected the mutation even though it occurs together with normal wild-type mtDNA. It has been previously demonstrated (Nickerson et al., 1997) that a minor allele can be detected when particularly abundant (it must be present in at least 30% of the sample to be detected by direct DNA sequencing of the PCR product). Additionally, in ICSI oocytes, even though a single spermatozoon contains ~100 mtDNA compared with 100 000–1 000 000 mtDNA in a human oocyte, this level of sperm contamination would not be detectable by chromatographic analysis. Only 4.4% of oocytes from patients between the ages of 26 and 36 (n = 11) exhibited this mutation. In contrast, 39.5% of oocytes from the older patient group (n = 10) harboured the T414G transversion. These data demonstrate a highly significant age-association for this mtDNA point mutation ($P < 0.01$). Further experiments are now in progress to determine the relative frequency of mutated mtDNA compared to wild type in the oocytes of older women.

Accumulations of mutations in the control region for replication of the mitochondrial genome may be involved in ageing. This 1000 bp mitochondrial region, including the hypervariable D-loop, contains the light strand promoter-
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Figure 3. Diagram of the mtDNA main control region adjacent to the hypervariable D-loop. The sites for heavy chain replication initiation (H-replication), light chain transcription (L-transcription), heavy chain transcription (H-transcription), light strand transcription promoter binding site (LSP-bs) and the conserved sequence boxes (1, 2 and CB3) are shown in the diagram. The mitochondrial mutation at position 414 is located in the binding domain of the light strand promoter (LSP).

Figure 4. Diagrammatic representation of T414G transversion mutation and transcription of mtDNA light chain. If a "T" nucleotide is present at base pair position 414 then the light strand promoter (LSP) binds and light-strand transcription proceeds. When a "G" nucleotide mutation is present at mtDNA base pair 414, the LSP may not bind, thus preventing light-strand transcription.

binding site (LSP-bs), light strand transcription start site, heavy strand transcription start site, as well as heavy (H) strand replication site (Figure 3). Additionally there are three evolutionarily conserved sequence blocks CSB1, CSB2 and CSB3. It has been postulated that nuclear transcription factors bind between these conserved regions and initiate transcription. The T414G transversion mutation occurs in the middle of the region where the light strand promoter (LSP) will bind. In particular mitochondrial populations that contain the mutation, G nucleotide at position 414, prevention of mRNA transcription of the light strand may occur (Figure 4). Similarly, the accumulation of this mutation as well as other mutations in the control region may be critical for mitochondrial replication. These regions may be more susceptible to oxygen radical damage because they must unwind and bend to allow all the nuclear transcription factors to bind the unprotected single–stranded DNA.

The association between light and heavy chain transcription, variation in mtDNA copy number and mitochondrial replication may be more complicated than ever imagined (Tang et al., 2000). Data from this laboratory suggest that there is quite a variation of mtDNA copy number (90 000–1 000 000) in individual human oocytes (Steuerwald et al., 2000; J Barritt, unpublished results). In particular, the degree of variation between samples and the concentration of mtDNA may indeed increase with maternal age. The high number of mitochondrial genomes may be attributed to a compensatory phenomenon or feedback mechanism, to guarantee sufficient ATP production in the event of cellular respiratory dysfunction. Perhaps the accumulation of high copy-number point mutations in the control region impairs mtDNA regulation as well as ATP synthesis, which in turn signals the cell to replicate the mtDNA, resulting in more mitochondrial genomes to compensate for mutated dysfunctional genomes.

The link between the accumulation of mitochondrial mutations, functional impairment of the ageing oocyte and the regulation of mitochondrial replication still remains to be elucidated. However, it is now known that the human oocyte harbours more than 23 mtDNA rearrangements in its small circular genome (Barritt et al., 1999). Also, the accumulation of these mutations may contribute to mitochondrial diseases such as Kears-Sayre syndrome as well as other neuromuscular
syndromes (Brenner et al., 1998). Most likely, the accumulation of specific point mutations in the mtDNA control region may be crucial for mitochondrial replication. Already, DNA sequence information from the hypervariable D-loop documents variation within and between human populations, allowing for the demographic history of human populations to be determined. Moreover, by using mitochondrial fingerprinting in the D-loop region, it has been possible to trace mitochondrial donor populations both during the ooplasmic transfer technique and in the bloods of the children (Brenner et al., 2000; J Barritt et al., unpublished results). Furthermore, adjacent to the D-loop, it has now been shown that a prevalent point mutation at position 414 in the mitochondrial genome may preferentially accumulate with age in the human oocyte. Although there is no evidence that any of these mutations functionally impair or change the rate of mitochondrial replication, these results give new ammunition linking mitochondrial mutations and ageing. "The mitochondria have now been compared to the fire of life," explains Vilhelm Bohr, a noted molecular biologist at the National Institute on Ageing. Perhaps that fire is slowly extinguished as we grow old.

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