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Spectrum of mitochondrial DNA deletions within the Common Deletion region induced by low levels of UVB irradiation of human keratinocytes *in vitro*

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Abstract

We show that a single low-dose exposure of human epidermal keratinocytes (NHEK) to an FS20 light source *in vitro* can induce the formation of mitochondrial DNA deletions in a PCR detection assay. We used primer sets specifically designed to exclude amplification of segments containing the common deletion, but which could detect possibly lower abundance deletions generated within the same region of the mitochondrial genome. We characterized eight novel deletions of which six were generated from cut sites within, or adjacent to, short direct repeats. Two deletions involved cut sites in inverted tetrameric repeats; one of these also involved an insertion.

Keywords

mtDNA deletion; inverted repeats; keratinocytes; UVB

1. Introduction

Mitochondrial DNA (mtDNA) deletions accumulate over time in a variety of tissues and are associated with diseases of heart, muscle, brain, and blood (reviewed in Filosto et al., 2007, Tabaku et al., 1999, Zeviani and Donauto, 2004). mtDNA deletions are generally believed to be the result of oxyradical-induced DNA damage but the mechanism of deletion is poorly understood. It is believed that mitochondrial DNA deletions and other mutagenic changes that affect the electron transport chain can bring about further DNA damage through the accumulation of reactive oxygen species (ROS) and, in this way, mitochondrial DNA damage may become a self-amplifying event (Krishnan et al., 2008).

A critical step in the formation of deletions appears to be slipstrand mispairing of nucleotide repeats (Schoffner et al., 1989). A 4977 bp deletion, known as the common deletion (CD), is produced from GC-rich 13 bp direct repeats at map locations 8470 and 13447. The CD is found in diverse tissue types and is the most frequently reported mtDNA deletion. We have previously demonstrated the induction of mtDNA deletions involving tetrameric repeats within the CD region in cultured normal human epidermal keratinocytes (NHEK) exposed to low levels of UVB radiation (Fang et al., 2006). Since a large number of nucleotide repeat sequences are

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located in this region of the mitochondrial genome, we hypothesized that oxidative agents such as UVB irradiation would probably produce a large number of deletions but that the majority of these are obscured in PCR reactions by deletions that are present at relatively high copy number. Here, we created a set of primers to scan the CD region of the mitochondrial genome in an effort to get a better picture of the range of deletions that is induced by UVB radiation in the NHEK *in vitro* model system.

2. Methods and Materials

Cultured normal human epidermal keratinocytes (NHEK) were irradiated with an FS20 lamp at 5.7 mJ/cm² as previously described (Fang et al., 2006). Deletion analysis was carried out essentially as described by Soong and Arnheim (1996) except that we chose to analyze only PCR products that were visible after a single round of amplification, without the need for a second round using nested primers. By employing a short 2-segment amplification protocol combining primer annealing and elongation at 60°C for 20 seconds, only short PCR products representing the deleted, rather than the intact mtDNA segments are amplified. For sequencing of PCR products, bands were cut from agarose gels and DNA was purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). PCR fragments were either sequenced directly or sequenced after cloning into a TA-cloning vector (Invitrogen, Carlsbad, CA). The locations of the deletion(s) boundaries were determined by alignment of the PCR product sequence with the Cambridge Mitochondrial DNA reference sequence (NC_001807; gi:17981852).

3. Results

We have previously described the formation of UVB-induced mtDNA deletions in human keratinocytes *in vitro* using an FS20 light source (Fang et al., 2006). In this system deletions are generated in a dose dependent manner even at low levels of UVB irradiation (Fig. 1). In the experiment shown in figure 1 the ML3/MR5 ampimer pair produces a 302 base pair PCR product that was seen after 4 minutes irradiation; sequence analysis confirmed that this product corresponded to the CD. A slightly larger PCR product that appeared after 8 minutes irradiation was found to represent a novel mtDNA deletion (Fig. 2C(ii)). We carried out a set of experiments using primer sets designed to scan for deletions along the CD region (Table 1). In these experiments we characterized at least 8 novel UVB-induced deletions of which 3 were found to be generated from cut sites at, or within, direct repeats (Fig. 2A). The repeat sequences in these deletions were between 7-8 nucleotides in length. Most of the deletions identified appear to be novel although one of these (Fig. 2A(ii)) was found to have been described previously in skeletal muscle (Zhang et al., 1995). The deletion shown in figure 2A(iii) may have been generated via cleavage at the 5' end of one of two imperfect heptameric direct repeats but could also have been formed by cleavage sites at sites within the repeats. Three deletions were generated from cut sites close to direct repeat sequences (Fig. 2B). Figure 2C details two unusual deletions one of which shows an insertion at a cut site within a 5'-aagt-3' and its inverted repeat (Fig. 2C(i)). The deletion in figure 2C(ii) represents the junction of cut sites within the complementary tetramers 5'-taat-3' and 5'-atta-3' but it is likely that formation of this deletion is mediated by the heptameric direct repeats (5'-tctcatc-3') adjacent to the cut sites.

4. Discussion

In standard PCR-based techniques used to detect mtDNA deletions, the presence of some mtDNA deletions can often be obscured by preferential amplification of PCR products from more abundant deletions. The experiments described here were instigated by the idea that mtDNA deletion-inducing stimuli rarely lead to the production of single deletions but rather induce the formation of a population of deletions many of which are not seen in routine assays. We explored the range of mtDNA deletions that are produced as a result of UVB irradiation

in an *in vitro* system of normal human epidermal keratinocytes. Our results underscore the utility of the keratinocyte system for studying mtDNA deletions. Unlike *in vitro* systems that employ fibroblasts which require multiple exposures to UV radiation over a period of time (Berneburg et al., 1999), mtDNA deletion formation in the keratinocyte system is highly sensitive to UVB irradiation.

Base pairing of repeat sequences is the key event that defines the deletion cut sites. It would therefore, appear that the number of possible deletions derived from short repeats is much greater than for relatively long repeats since short repeats occur, on average, more frequently in the genome. For this reason, it seems likely that deletions derived from short repeats would comprise a relatively large, and probably more heterogeneous population than those derived from longer repeats. The distribution of mtDNA deletions tabulated in the Mitomap (2008) deletion database is in good agreement with this prediction (Fig. 4). Deletion cut sites are most frequently found at direct repeat sequences of between 5 and 6 nucleotides in length. For longer repeats, there is a steady decrease in the number of deletions as the length of the repeat sequences increases. The smaller number of deletions for repeats that are shorter than 5 nucleotides may reflect a lowered ability to form stable duplexes.

This raises the possibility that for every mtDNA deletion that is detected using a single amplicon pair, there are probably a large number of deletions present that are not detected using standard PCR techniques. Seven out of the 8 mtDNA deletions described here were associated with direct repeats. This is consistent with the general observation that direct repeats appear to mediate the large majority of mitochondrial deletions reported in the literature. It is generally believed that direct repeats mediate the formation of deletions by slipstrand mispairing of the repeats followed by recombination at cut sites within the repeats. The deletion shown in 2C(i) is a much less common variant which contains an insertion at the junction between inverted repeats. Inverted repeats may also be involved in the formation of this type of deletion through a base pairing mechanism in which the repeat sequences are aligned to one another in a parallel orientation as occurs in triple helices (H-DNA). Since this type of structure is probably incompatible with normal mechanisms of recombination, deletions derived from inverted repeats likely occur by a somewhat different mechanism. Template strand switching during DNA replication presents a possible alternative. There is good evidence for deletion formation caused by template strand switching at segments containing both direct and palindromic repeats in viral and yeast systems (Cheung, 2004, Lewellyn and Loeb, 2007, Nag et al., 2005) as well as in bacteria (Pinder et al., 1998) and mitochondria (Mita et al., 1990). Figure 3 illustrates a scheme for generating mitochondrial DNA deletions by a strand switching mechanism. The scheme also illustrates a possible mechanism for generating the insertion seen in fig. 2C(i). Single or dinucleotide insertions could be generated by any of a number of mechanisms. But we noted that the sequence of this deletion is consistent with insertions known as P insertions that are generated from hairpin intermediates during the process of VDJ **recombination** (Lewis, 1994). We show a hairpin structure that would be generated by joining the ends of complementary DNA strands that could produce the observed insertion by a similar mechanism.

The number of deletions produced by irradiation under the conditions used here is quite small. We have previously shown that, in this system, the radiation-induced levels of the common deletion were about 0.013 copies/cell or roughly 0.12% of the mitochondrial genomes (Fang et al., 2006). Estimates of the deletions described here by real time PCR ranged from about 10^{-5} (for the deletion in Fig.2 B(i)) to 4×10^{-4} (for the deletion in A(i)) deleted per every undeleted mitochondrial genome (not shown).

It has been proposed that subpopulations of damaged mitochondrial genomes may undergo spontaneous expansion so that, over time, their numbers may greatly increase in number

relative to their intact counterparts. Alterations that affect genes of one or more of the carriers in the electron transport chain can lead to accumulation of electrons in the unaffected upstream carriers along the chain. In this scenario, electrons transfer to O₂ to form a short-lived superoxide radical (O₂^{·-}) and this can, either directly or indirectly (via the formation of intermediate H₂O₂), form DNA damaging hydroxyl radical (·OH). This model also explains how even low levels of mtDNA alterations can become self-amplifying since hydroxyl radicals could accumulate to high concentrations locally within the mitochondrial compartment causing further damage to the mtDNA (this model is reviewed in: Birch-Machin, 2006, Balaban et al., 2005). The deletions described here all encompass segments that lead to the loss of genes for complexes I, III, IV and V but not of the mitochondrially encoded cytochrome b.

It has also been suggested that some mutation-bearing mitochondrial genomes can undergo clonal expansion as a result of selective replication (Yoneda et al., 1992, Elson et al., 2001). This mechanism has been proposed to explain why, in certain mitochondrial disease states, unique mitochondrial mutations predominate in a large proportion of the cells of the affected tissues. From this standpoint it is noteworthy that one of the deletions uncovered in our screen was previously shown to be associated with chronic muscle fatigue syndrome (Zhang et al., 1995). This raises the possibility that many deletions of potential significance may not be detected in studies that rely on single amplicon pairs that target specific deletions.

5. Conclusions

We employed a human keratinocyte model system to study the generation of mitochondrial DNA deletions in response to UVB irradiation. Sequence analyses of PCR products containing the deletion cut sites suggested that, mtDNA deletions may be formed by mechanisms that involve base pairing of short repeat sequences in both parallel and antiparallel orientations. In the latter case deletions would likely be produced by a mechanism(s) that differs from simple slipstrand mispairing as is seen to occur when cut sites are within direct repeats. For antiparallel base pairing of inverted repeats, template strand switching during the DNA repair process offers a possible mechanism for generating mtDNA deletions.

Acknowledgments

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Figure 1.

Dose dependent induction of mtDNA deletions by UVB irradiation of NHEK. NHEK cells were subjected to various doses of UVB radiation with an FS20 light source as indicated. Using primers ML3 and MR5 PCR products of 302 and 391 bp corresponding to the common deletion (CD) and a novel deletion (detailed in Figure 2C(ii)) respectively are visible 24 hours after a 4 or 8 minute irradiation ($1.43 \text{ mJ/cm}^2/\text{min}$) with an FS20 light source.

Figure 2.

mtDNA deletion cut sites. Mitochondrial nucleotide numbering according to the revised Cambridge reference sequence (HUMMTTCG J01415.2 gi:113200490). Locations of cleavage sites in mitochondrial genomic sequences are indicated (▼ or ▲). Nucleotide repeats at or near the sites of cleavage are bracketed. In A, the placement of ▼ above the bracket indicates that the exact cleavage site within the nucleotide repeat is unknown. The joined ends in the deletion are indicated by ∇. The deletions in A are formed by cleavage within direct repeats. The deletion in iii is an imperfect repeat. Deletions formed by cleavage near, but not within, repeats are shown in B; nearby repeats adjacent to the cut sites are bracketed. The box in the deletion in C(i) shows a dinucleotide insertion. The primer pairs (Table 1) used to generate the various PCR products are: A(i):ML2/MR1, A(ii):ML4/MR3, A(iii):ML6/MT3, B(i):MT1A/MT2, B(ii):MT1A/MT3, B(iii):ML4/MT3, C(i):MT1A/MT2, C(ii):ML3/MR5

Figure 3.

A strand switching scheme for the formation of the deletion shown in figure 2C(i). Irradiation produces a single strand nick between the upstream (5') and downstream (3') repeats. (1) Degradation of the nicked strand by the action of 3' → 5' exonuclease past the 5' repeat produces a single stranded segment containing the complementary 5' repeat (Krishnan et al., 2008). (2) Slipstrand base pairing between the 5' and 3' repeats. Since the repeats are inverted strands would be aligned in parallel in a triple helical configuration. (3) A hairpin is formed during DNA replication that is initiated from the recessed 3' end. (4) Asymmetric resolution of the hairpin (the cleavage site is indicated by the arrowhead) followed by DNA replication during

which template strands are switched (5) to produce an mtDNA that contains both the deletion and the nucleotide insertions (6).

Figure 4.

Frequency analysis of mitochondrial DNA deletions in the Mitomap (2008) database. Mitochondrial DNA deletions tabulated on the Mitomap website database were categorized according to the length of the nucleotide repeats at the deletion cut site.

Table 1

Primers used to amplify mitochondrial DNA segments containing deletions

Primer	Primer sequence	mitochondrial nucleotides
Forward primers		
^a MT1A	gaattcccctaaaaatctttgaaat	8225 → 8247
ML2	tacccctctagagcccact	8280 → 8299
ML3	cccctctagagcccactgta	8283 → 8302
ML4	atggcccaccataattacc	8390 → 8409
ML6	ccctctattgatccccacct	8608 → 8627
Reverse primers		
^a MT2	Aacctgtgaggaaaggtattcctgc	13478 → 13502
^a MT3	gcgatgagagtaataagataggctcaggcg	13552 → 13581
MR1	ttggttgatccgattgtaa	13281 → 13300
MR3	tcgatgatgtggtctttgga	13508 → 13527
MR5	caggaggtagcgtatgagag	13572 → 13591

^aMT1A, MT2, and MT3 are from Soong and Arnheim (1996)