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Mutation Research 585 (2005) 127–136
Genetic Toxicology and Environmental Mutagenesis
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Mitochondrial DNA point mutations and a novel deletion induced by direct low-LET radiation and by medium from irradiated cells

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Received 4 November 2004; received in revised form 19 April 2005; accepted 26 April 2005

Abstract

Radiation damage incurred by nuclear DNA is well documented and interest is increasing in the properties of 'bystander' factor(s) and their ability to induce radiation-like damage in cells never exposed to radiation. 'Bystander' and direct low-LET radiation effects on the mitochondria, and more particularly the mitochondrial genome are less well understood. In this study HPV-G cells (a human keratinocyte cell line derived from human neonatal foreskin transfected with the HPV-16 virus) were exposed to either γ-radiation doses as low as 5 mGy and up to 5 Gy from a ^{60}Co teletherapy unit, or to growth medium taken from similarly irradiated cells, i.e. irradiated cell conditioned medium (ICCM). Mutation and deletion analysis was performed on mitochondrial DNA (mtDNA) 4–96 h after exposure. Primers flanking the so-called mitochondrial 'common deletion' were employed to assess its possible induction. Single-strand conformation polymorphism (SSCP) analysis was conducted to identify induced point mutations. The relative mitochondrial number per cell was analysed by semi-quantitative PCR (sqPCR). Results indicate the induction of a relatively novel deletion in the mitochondrial genome as early as 12 h after direct exposure to doses as low as 0.5 Gy and 24 h after exposure to 0.5-Gy ICCM. SSCP analysis identified the induction of point mutations, in a non-consistent manner, in only the D-loop region of the mitochondrial genome and only in cells exposed to 5 Gy, and neither in cells exposed to lower doses of direct radiation nor in those exposed to ICCM. SqPCR also identified an increase in the number of mitochondria per cell after both exposure to low level γ-radiation and ICCM, indicative of a possible mechanism to respond to mitochondrial stress by increasing the number of mitochondria per cell.

Keywords: Mitochondrial DNA; Radiation; Bystander; Mutation; Novel deletion

1. Introduction

It is widely accepted that cancer may be induced by radiation through accumulation of DNA damage in genes encoding proteins that regulate the cell cycle. To

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doi:10.1016/j.mrgentox.2005.04.011

date there has been a variety of radiation levels suggested as the maximum 'safe' exposure dose. More recently, it is becoming apparent that there may indeed be no such thing as a 'safe' level of radiation exposure. This would concur with the characterisation of the 'bystander' effect. This is a mechanism whereby cells, never exposed to radiation, display radiation-like damage if in the vicinity of irradiated cells [1–3]. More importantly, the extent of this effect remains the same no matter how low the radiation dose may be. Evidence suggests that at very low doses of radiation the damage induced is predominantly a result of the release of 'bystander' factor(s) and the direct radiation effects are indeed negligible [4]. The phenomenon of 'bystander' effects can be examined in one of several ways. Cells may be exposed to alpha particles such that only a small percentage of cells are actually traversed [5]. A micro-beam may be employed to traverse a single cell in a population of cells [6]. Finally, the method employed in this study, a flask of cells may be irradiated, the medium removed, filtered, and applied to a flask of cells never before irradiated [2,7].

The mitochondrion is the only other location of genetic material outside of the nucleus. Although the mitochondrial genome encompasses a minute fraction of the total genetic material in a cell, any damage or alteration to it can still have serious implications for a cell's viability and/or survival. Indeed, mtDNA is more prokaryotic in nature than eukaryotic in employing prokaryote-like codons and having no histone coat. MtDNA is almost entirely made up of coding regions, including some overlapping genes, increasing the potential of any point mutation or deletion to have a subsequent phenotypic effect. The genome is also located in close proximity to a significant potential source of free radicals, as electrons are passed from carrier to carrier on the electron transport chain embedded in the inner mitochondrial membrane. The mtDNA polymerase machinery also possesses limited proofreading ability and all of these peculiarities of the mitochondrial genome suggest why there is a 10- to 20-fold greater mutation rate in this genome than in the nuclear genome.

Many point mutations in the mitochondrial genome are associated with severe disorders, such as the mutations A3243G inducing mitochondrial encephalomyopathy and lactic acidosis with stroke-like episodes (MELAS) [8–10] and A8344G inducing myoclonic epilepsy with ragged-red fibre (MERRF) [11,12]. The mitochondrial genome and the damage it accumulates provide a useful marker of the effects of many stress factors and disease types. MtDNA damage has been found to be associated with many tumour types and stress factors, such as Warthin's tumour in the salivary glands [13], radiation-associated thyroid tumours [14,15], hepatocellular carcinoma [16] bladder cancer [17], squamous-cell carcinomas [18] and UVA-induced damage [19]. The 'common deletion' is a 4977-bp deletion occurring between two 13-bp repeats from position 8470–13,447 [20–23]. The deleted mtDNA segment contains all or part of the genes encoding seven polypeptides and five tRNA genes. As its name suggests, it is the most frequently reported deletion associated with mitochondrial damage. It is prevalent in such disorders as Kearns Sayre Syndrome [24], it is found to accumulate with age [25], to be induced by X-ray exposure [26] and found associated with several tumour types [15,19,27]. There is however, increasing evidence that this common deletion, though frequent, is not necessarily accumulating with age and several less 'common' deletions show accumulation with age and/or insult and may represent better markers of mitochondrial stress [28,29]. Damage accumulation remains a well established marker of human ageing [25,30–32], although due to the multiple copy number per cell most point mutations and deletions may accumulate to a relatively significant frequency in the mitochondria before an effect is manifest in the cell and/or tissue. This study aims to provide an in-depth evaluation of the extent of damage incurred by mtDNA after direct exposure to low level γ-radiation and bystander factor(s), and of the potential for damage potentiation with time.

2. Materials and methods

2.1. Culture of HPV-G cells

HPV-G cells (a keratinocyte cell line derived from human neonatal foreskin transfected with the HPV-16 virus) were maintained in Dulbecco's Modification of Eagle Medium and Ham's Nutrient Mixture (1:1) supplemented with 10% foetal calf serum, 20 mM

L-glutamine, 1 U/ml penicillin/streptomycin and 1 μg/ml Hydrocortisone. Cells were maintained until 70–80% confluency and subsequently passaged or exposed to either direct radiation or ICCM, described below.

2.2. Irradiations and exposure to 'bystander' factors

HPV-G cells were irradiated with either a dose of 5 mGy, 0.5 Gy or 5 Gy γ-radiation from a ^{60}Co teletherapy unit (St. Luke's Hospital, Rathgar, Dublin 6, Ireland) that employed a dose rate of 1.8 Gy/min with a source-to-flask distance of 80 cm. Control cells were sham-irradiated by undergoing the same routine as treated cells save exposure to radiation. Cells were harvested 4 to 96 h after irradation in the direct radiation studies. For 'bystander' studies, medium from irradiated cells was removed 1 h after exposure, passed through a 0.2-μm sterile filter and added to flasks of previously unirradiated cells. Earlier studies confirmed the presence of bystander factor(s) in culture medium as soon as 30 min after irradiation (2). Controls were exposed to filtered medium from sham-irradiated cells. Cells were similarly harvested 4–96 h later. Cells were maintained after treatment in normal cell-culture conditions until harvested.

2.3. DNA isolation, quantification, separation and visualisation

Total DNA was isolated using the Total DNA GenElute kit (Sigma) and following the manufacturer's instructions. DNA purity and concentration were confirmed by spectrophotometric analysis at 260 and 280 nm. PCR products were electrophoresed on a 1.0–1.2% agarose gel embedded with ethidium bromide. Bands were visualised, photographed, and band densities calculated using a GeneGenius DNA Imager and associated software (Syngene).

2.4. MtDNA deletion analysis

A pair of primers designed to flank the region of the mitochondrial genome where the common deletion occurs were employed. The forward primer (8151) cgggggtatactacggtcaa and the reverse primer (13509) ggtttcgatgatgtggtctttg amplify a 393-bp fragment from mitochondrial genomes incorporating the common deletion, the limited elongation time preventing amplification in the wild-type genome. Primers would amplify a 5370-bp product from wild-type genomes given sufficient elongation time. PCR reaction volumes (25 μl) contained 1× Ready Mix PCR Buffer (Sigma; including 1.5 mM $MgCl_2$, 0.5U Taq polymerase and 4 μM dNTPs), 0.1 μM of each primer and 5 ng DNA. Reactions were put through 38 cycles of 94 °C for 30 s, 54 °C for 1 min and 72 °C for 1 min in a Techne thermal cycler. Initially, PCR reactions were terminated after 32, 35, 38 and 41 cycles to confirm that a cycle number of 38 remained within a linear amplification range. PCR products were visualised as described in Section 2.3.

2.5. Amplicon sequencing

PCR deletion analysis products were visualised as described in Section 2.3 and bands indicating amplification across a deletion in the mitochondrial genome were excised from the gel. DNA was extracted and purified from the agarose using the QiaQuick kit (Qiagen) and following the manufacturer' instructions. DNA fragments were then sent to MWG Biotech, Ebersberg, Germany for sequencing.

2.6. Semi-quantitative PCR analysis

A small 526-bp region of the mitochondrial genome, known to be unaffected by most deletions and away from point-mutation hotspots, was amplified as a marker for mitochondrial genome frequency using the primers cttaccacgctactcctacc (5463) (forward) and aggactccagctcatgcgcc (5989) (reverse). A small region of the nuclear genome (155 bp) encoding the 18S RNA gene was amplified as a control, using the primers gtaacccgttgaaccccatt (forward) and ccatccaatcggtagtagcg (reverse). PCR reaction volumes (25 μl) contained 1× Ready Mix PCR Buffer (Sigma) (which includes 1.5 mM $MgCl_2$, 0.5U Taq polymerase and 4 μM dNTPs), 0.1 μM of each primer and 5 ng DNA. Reactions were put through 30 cycles of 94 °C for 40 s, 56 °C for 1 min and 72 °C for 40 s. Initially, PCR reactions were terminated after 25, 30, 35 and 40 cycles to confirm that a cycle number of 30 was within a linear amplification range.

Table 1
Primers employed in SSCP analysis

Primer set	Forward primer	Reverse primer	Location	Size
H1	aatcaacaaaactgctcgcca	gcgtattcgatgttgaagcct	1117–3953	2836
H2	tagcagagaccaaccgaacc	aacctgttcctgctccgg	3875–6279	2404
H3	tctctcctactcctgctcgc	cagatttcagagcattgaccg	6225–8184	1959
H4	actccttgacgttgacaatc	ctacaaaatgccagtatcagg	7986–9942	1956
H5	gtctcccttcaccatttccg	gcaagtactattgacccagcg	9755–11454	1699
H6	tacctgactcctacccctcac	ggattagcgtttagaagggct	10966–12959	1993
H7	gagcagatgccaacacagc	cgtgtgagggtgggactg	12852–15276	2424
H8	ggggcagacctagttcaatga	agcaagaggtggtgaggttg	15215–1249	2603

PCR products were visualised as described in Section 2.3.

2.7. Analysis of single-strand conformation polymorphism

The entire mitochondrial genome was amplified as eight overlapping fragments (Table 1). PCR reaction volumes (50 μl) contained 1× PCR Buffer (Sigma), 1 U Taq DNA polymerase (Sigma), 4 μM dNTPs mix, 0.1 μM of each primer (Table 1) and 5 ng DNA. Reactions were put through 30 cycles of 94 °C for 40 s, 54–56 °C for 1 min and 72 °C for 3 min. PCR products were visualised as described in Section 2.3. Products were restriction-digested overnight by 1 U of enzyme(s) (Table 2). An equal volume of 10 mM EDTA, 1 mM NaOH, 0.1 mg/ml xylene cyanol and 0.3 mg/ml bromophenol blue in formamide was added to digested fragment samples. Samples were then boiled for 10 min and chilled rapidly to convert fragments to single-stranded DNA (ssDNA), which was then separated on an 8% acrylamide gel overnight and silver-stained according to Bassam et al. [33].

3. Results

3.1. MtDNA deletion analysis

Total DNA was extracted from HPV-G control cells and from cells 96 h after irradiation with 5 mGy, 0.5 Gy and 5 Gy, as well as from cells 96 h after exposure to 5-mGy, 0.5-Gy and 5-Gy ICCM (bystander effects). An equal amount of DNA was added to each PCR reaction and amplification was only achieved from template containing a deletion. No amplified product was observed from control HPV-G DNA, however, pronounced amplification was achieved from DNA extracted from cells irradiated with 5 mGy, 0.5 Gy and to a lesser extent 5 Gy and also from cells exposed to 5-mGy, 0.5-Gy and 5-Gy ICCM (bystander effects). This result shows pronounced accumulation of a deletion 96 h after treatment in both directly irradiated and bystander-treated cells compared with control cells (Fig. 1A. DNA was also extracted from HPV-G cells 4, 12, 24 and 96 h after irradiation with 0.5 Gy and after exposure to 0.5-Gy ICCM. Pronounced amplification of product was first seen in samples from directly irradi-

Table 2
Restriction enzymes employed in SSCP analysis

Primer Set	Location	Size	Restriction enzymes	Predicted fragment sizes
H1	1117–3953	2836	AflIII, ApaI, HpaII	1090, 695, 521, 352, 168
H2	3875–6279	2404	EcoRI, HaeIII	1004, 741, 412, 247
H3	6225–8184	1959	XbaI, EcoRV	742, 704, 513
H4	7986–9942	1956	HinfI, HaeIII	797, 697, 273, 189
H5	9755–11454	1699	HaeIII	728, 611, 325, 35
H6	10966–12959	1993	HpaII	834, 724, 435
H7	12852–15276	2424	AvaI, EcoRII	852, 798, 453, 321
H8	15215–1249	2603	HpaII	829, 712, 529, 313, 220

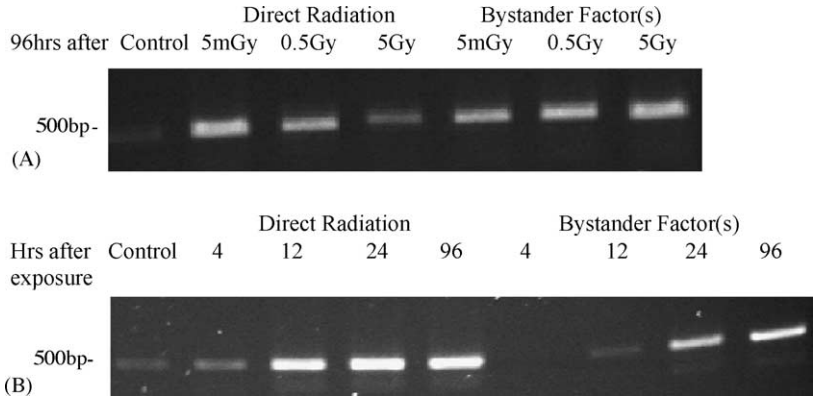


Fig. 1. A novel mtDNA deletion was identified using primers that flank the region of the genome affected by the 'common deletion' and employing PCR parameters such that amplification is achieved from genomes with a significant deletion. Primer loci are 5370 bp apart in a wild-type genome. (A) DNA was isolated from HPV-G cells 96 h after γ irradiation with 5 mGy–5 Gy or after exposure to medium from cells similarly exposed. (B) DNA was isolated from HPV-G cells 4, 12, 24 and 96 h after 0.5 Gy γ-radiation or after exposure to 0.5-Gy ICCM. Images are representative of duplicate experiments.

ated cells 12 h after exposure, with a further increase at 24 and 96 h (Fig. 1B). Significant amplification of product was first seen in samples from cells 24 h after exposure to ICCM, with a further increase at 96 h (Fig. 1B). Indeed, the lower doses of direct radiation, such as 5 mGy and 0.5 Gy appear more potent than the 5-Gy dose in inducing this deletion and 5-mGy, 0.5-Gy and 5-Gy ICCM all appear equipotent (Fig. 1A). The amplicons identifying a deletion were approximately 500 bp in size when a 393-bp fragment was predicted from the common deletion. Subsequent DNA sequencing (MWG Biotech, Germany) confirmed one consistent mtDNA deletion though not the 4977 bp common deletion as expected, but a relatively novel 4881 bp deletion [34] flanked by a 7 bp repeat (Fig. 2) that produced a 499-bp fragment. This deletion was confirmed using

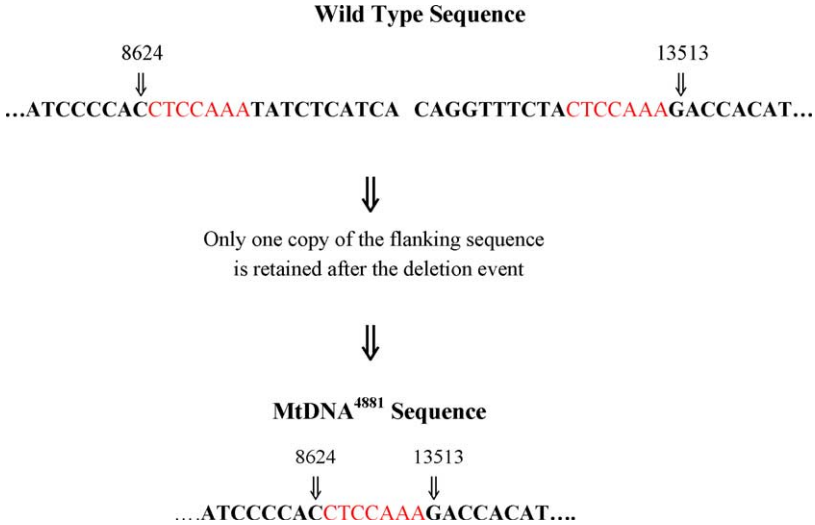
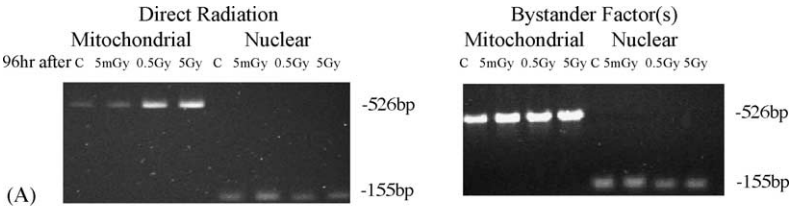


Fig. 2. Characterisation of a novel mtDNA deletion was performed by excising the identified band from an agarose gel, purifying the DNA, resuspending in water and DNA sequencing (at MWG Biotech, Germany). This revealed a 4881-bp deletion in the mitochondrial genome. Bases highlighted in red indicate the 7-bp flanking sequences.



96 Hours after	Direct Radiation	Bystander
Control	100	100
5 mGy	138 ±12	166 ±5
0.5 Gy	295 ±39	170 ±22
5 Gy	317 ±53	194 ±19

Hours after 0.5Gy	Direct Radiation	Bystander
Control	100	100
4	141 ±3	98 ±8
12	128 ±7	116 ±11
24	154 ±12	176 ±12
96	191 ±25	205 ±28

(C)

Fig. 3. qual amounts of DNA (mtDNA + nDNA) were added to each PCR reaction. A small region of the mitochondrial genome, known to be largely unaffected by mutations or deletions was amplified after various exposure conditions described above. (A) DNA was isolated from HPV-G cells 96 h after irradiation with 5 mGy, 0.5 Gy and 5 Gy γ-radiation and after exposure to 5-mGy, 0.5-Gy and 5-Gy ICCM. (B) DNA was isolated from HPV-G cells 4, 12, 24 and 96 h after irradiation with 0.5 Gy γ-radiation and after exposure to 0.5-Gy ICCM. A region of the nuclear 18S RNA gene was amplified as a standard each time. Images are representative of duplicate experiments. The tables (C) show the mean density of mitochondrial bands as a percentage of the control, with errors expressed as standard error of the mean.

2 other primer sets, including nested primers and subsequent DNA sequencing (MWG Biotech, Germany) (result not shown).

3.2. Semi-quantitative PCR analysis

Total DNA was extracted from HPV-G control cells and from cells 96 h after irradiation with 5 mGy, 0.5 Gy and 5 Gy as well as from cells 96 h after exposure to 5-mGy, 0.5-Gy and 5-Gy ICCM (bystander effects). An equal amount of DNA was added to each PCR reaction. Cells irradiated with 0.5 Gy and 5 Gy showed a pronounced increase in the number of mitochondria per cell 96 h after exposure (Fig. 3A and C), an increase >2-fold that of control cells. DNA was also extracted from HPV-G cells 4, 12, 24 and 96 h after irradiation with 0.5 Gy and after exposure to 0.5-Gy ICCM. An increase, with time, in the number of mitochondria per cell was observed after these treatments. This increase was pronounced at 24 h after exposure and reached a level of approximately 2-fold that of control cells at 96 h (Fig. 3B and C). Amplification of a fragment of the 18S RNA sequence was used as a nuclear standard each time.

3.3. Analysis of single-strand conformation polymorphism

Total DNA was extracted from HPV-G control cells and from cells 4, 8 and 24 h after irradiation with 5 mGy, 0.5 Gy and 5 Gy, and after exposure to 5-mGy, 0.5-Gy and 5-Gy ICCM (bystander effects). An equal amount of DNA was added to each PCR reaction. The entire mitochondrial genome was subjected to SSCP analysis initially through the amplification of the genome as eight overlapping fragments. Only one fragment was found to be susceptible to mutations, the region denoted H8 (Table 1), which entirely encompasses the D-loop region. Indeed, although the mutation loci were found to be variable, their reoccurrence in this hot spot was reproducible. Only cells irradiated with 5 Gy showed the presence of

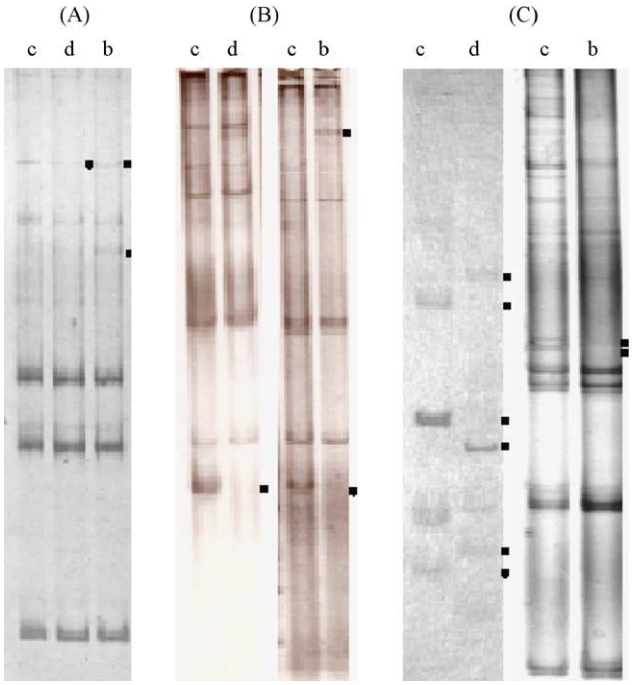


Fig. 4. SSCP analysis of mtDNA from cells harvested (A) 4 h (B) 8 h and (C) 24 h after irradiation with 5 Gy γ-radiation or after exposure to 5-Gy ICCM. DNA was isolated and 2–3-kbp fragments amplified by PCR, restriction-digested and converted to ssDNA. ssDNA fragments were separated on an 8% acrylamide gel and silver-stained. Lanes marked 'c' are samples derived from control cells, lanes marked 'd' are samples derived from directly irradiated cells and lanes marked 'b' are samples derived from cells exposed to bystander factor(s) (ICCM). Polymorphic banding is denoted by a black dot. Images are representative of duplicate experiments.

point mutations in the mitochondrial genome 4, 8 and 24 h after exposure to both direct radiation and medium from 5Gy-irradiated cells (Fig. 4). Mutations appeared most abundant in cells 24 h after irradiation with 5 Gy (Fig. 4). SSCP analysis of regions H1–H7 (Table 1) showed no observable induction of point mutations (data not shown) at any radiation dose or ICCM treatment. SSCP analysis performed on cells exposed directly to both 5 mGy and 0.5 Gy as well as to 5-mGy and 0.5-Gy ICCM showed no observable point mutations in region H8 (data not shown).

4. Discussion

'Bystander' factor(s) have the ability to induce mtDNA damage with efficacy similar to that of direct radiation in HPV-G cells at doses as low as 5 mGy and as soon as 12 h after exposure. Damage to the mitochondrial genome from low level γ-radiation and ICCM has been shown in this study to include point mutations and the induction of a relatively novel deletion. The response of cells containing damaged mtDNA appears to be to increase the number of mitochondria per cell as is suggested by the findings presented in this study. This would compare favourably with such findings as those of Limoli et al. [35], who found elevated numbers of mitochondria in cells suffering from mitochondrial dysfunction resulting from radiation exposure. Maguire et al. [36] has also recently shown elevated numbers of mitochondria in cells after exposure to ICCM. It is thought that dysfunctional mitochondria may be at a replicative advantage resulting from either a depleted genome replicating faster or the formation of new damaged organelles [37–39]. However, Kowald [40] has previously suggested that cells with dysfunctional mitochondria tend to have elevated numbers of mitochondria per cell due to a delayed degradation rate and not as a result of a cellular response that up-regulates mitochondrial proliferation nor as a result

of reduced replication time of a depleted genome. The increase in the number of mitochondria observed in this study is also a likely result of a stress response that upregulates mitochondrial proliferation, given the short time scale in which the increase was observed.

This study has also shown an increase in the frequency of a relatively novel deletion with time after exposure to radiation and 'bystander' factor(s). The fractions of genomes incorporating the deletion are seen to increase with time after both direct irradiation and exposure to ICCM, although the response is more immediate after direct irradiation. It must also be noted that in the case of direct irradiation, lower doses appear more potent although ICCM derived from the three radiation doses employed appear equipotent. This would concur with trends seen in previous analyses of low-level radiation and bystander effects, namely that the strength of the bystander effects appears similar irrespective of the original exposure dose, and that the lower dose of direct radiation is often the more potent [4]. This increase in the frequency of genomes reduced in size by 30% could be due either to the ability of the DNA replication machinery to copy this reduced genome faster than an intact genome [30] or to a reduced degradation rate of mitochondria containing damaged genome(s) [40]. The deleted region includes genes encoding ATP synthase F0 subunit 6, Cytochrome c oxidase subunit III, NADH dehydrogenase subunit 3, NADH dehydrogenase subunit 4L, NADH dehydrogenase subunit 4, NADH dehydrogenase subunit 5 and 5 tRNAs. Should the accumulation of this deletion continue, the implications would be grave for the viability of the distal progeny of these cells. This is a relatively novel deletion and to our knowledge, there has been only one previous report of this deletion [34]. It is obvious then that given the proximity of this deletion to the common deletion and the fact that it was identified using primers chosen to flank the common deletion, the sequencing of the PCR products remains essential and indeed even the use of nested primers to identify the common deletion may now be called into question if not also accompanied by DNA sequencing. The induction of the common deletion in primary fibroblasts by ionising radiation has been reported previously by Prithivirajsingh et al. [41], although the minimum dose at which effects were examined in this study was 2 Gy and cells were shown to have pronounced radiosensitivity.

The trend of deletion accumulation is not the same for all cell types, however, and appears to predominate only in cell types that are slowly dividing, such as in muscle and nervous tissue [42–44]. Koch et al. [19] found that the frequency of the common deletion induced by UVA in keratinocytes reduced with time after exposure. Indeed the common deletion may not be the best marker of human ageing or mitochondrial damage it was once thought to be, as was also suggested by Bodyak et al. [28] and Thayer et al. [45]. The authors found other deletions more abundant than the common deletion in many tissues, suggesting perhaps it is not so deserving of the title 'common'. The trend that mitochondria containing deleted mtDNA increase in frequency with time compared with wild-type mitochondria [46] would suggest that once a mitochondrion contains one or more reduced genomes, it is only a matter of time before a threshold fraction of genomes are similarly affected and the efficiency of that cell and its progeny compromised.

The initiation of point mutations in the mitochondrial genome has been established in such conditions as prostate cancer [47] and in cells following UVC exposure [48]. Mutations were found in this study to be induced in the mitochondrial genome by both direct irradiation with 5 Gy and by exposure to bystander factor(s) obtained from cells exposed to 5 Gy, though not by lower doses of direct exposure or ICCM. We found one area of the genome that could be considered a 'hot spot' of mutation induction, namely the region covered by primer set H8, which includes the D-loop region of the genome. This finding correlates with what has been well reported recently, namely that the induction of point mutations in mtDNA predominates in the D-loop region [49–51]. Mutations were not directed to specific loci, as replication of the induction of a mutation at the same locus proved difficult although reproduction of mutation induction in the same genomic region was achieved, demonstrating the random nature of point mutations initiated in this region.

This study has served to further underline the heretofore underestimated damaging properties of low-level irradiation and the significant role played by bystander factor(s) in effecting this damage. The findings also have serious implications for the long-term viability of mitochondria contained in the distal progeny of exposed cells.

Acknowledgements

This work was funded by Science Foundation Ireland. We also wish to thank St. Luke's Hospital, Rathgar, Dublin for their continuing co-operation.

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