

Mitochondrial Apoptosis Reduces Mutagenesis Regardless Oxidative Stress

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Abstract

Upon oxidative stress DNA accumulates adducts and breaks that activate the genome damage response to repair, arrest and eventually suicide the damaged cell. Indeed, challenges with exogenous pro-oxidants increase mutations and reduce survival. Thus, the amounts of pro-oxidants generated by endogenous oxygen metabolism are thought to affect mutation frequency. However, oxidative stress induces cell death, clearing damaged cells.

The LacZ reporter system has been used previously to detect mutation rate in flies and mice. Recently, we have measured *in vivo* spontaneous mutation rate in mice with reduced mitochondrial ROS production and cell death rate, by crossing p66Shc or Cyclophilin D knockout mice, characterized by reduced intracellular concentration of reactive oxygen species and by impaired apoptosis, with a transgenic line harboring multiple copies of the LacZ mutation reporter gene.

Results indicated that the inhibition of endogenous oxidative stress and the following induced apoptosis increased genome rearrangements suggesting that specific genetic sets, in precise environments, determine somatic mutations rate.

Keywords: Mutagenesis; Reactive oxygen species; Apoptosis; Mitochondria

Introduction

DNA is vulnerable to an array of damaging agents of both, endogenous and environmental origin. Therefore, specialized DNA repair systems evolved to preserve nuclear DNA integrity. These systems are continuously required for the excision and replacement of damaged nucleotide residues. Indeed, if DNA damage is not promptly and faithfully repaired, DNA replication can fix the mutation. When levels of DNA damage are elevated, DNA repair systems become overwhelmed and mutations accumulate. The level of DNA repair activity declines with age [1] therefore the mutational burden of the cell increases as organisms become older [2]. Genomic instability is considered a major stochastic mechanism of ageing [3,4]. Moreover, accumulation of high mutation rates may lead to cell death, cancer, premature ageing and neurodegenerative diseases [5].

Somatic mutations, point mutations (transitions and transversions) and chromosome rearrangements, can be generated by an endogenous or an exogenous source of DNA damage. There is a broad variety of environmental factors that can cause the accumulation of mutations in living organisms, among them the chemicals formed during the combustion of sulfur-containing fossil and N-methyl-N-nitrosamine, present in tobacco smoke (Swann and Magee, 1968), mutagens present in food such as aflatoxins and UV radiation.

Reactive Oxygen Species (ROS) are highly reactive products of oxygen (O₂) reduction and include superoxide anion (O₂^{•-}), hydrogen peroxide (H₂O₂) and hydroxyl radical (OH[•]). When high-energy radiations, such as X rays and gamma radiations, interact with water, ROS can be formed. They are also produced during normal cell metabolism by specific proteins. Life on earth is largely based on oxygen metabolism that is the driving force for the majority of reactions occurring in living organisms. As a general rule, reductive processes are used to store energy and oxidative processes to release it. Complete O₂ reduction produces water, whereas its partial reduction produces ROS. The high reactivity of ROS is due to the presence of unpaired

valence shell electrons. Hence, ROS can alter proteins and lipids as well as DNA. ROS cause modification of purine and pyrimidine bases, of the deoxyribose backbone, single and double strand breaks, as well as cross-links to other molecules. More than 20 different types of base damage have been identified. The most frequent damage to purines produces the formation of 8-oxo-dG (7,8-dihydro-8-oxoguanine), while for pyrimidines the formation of thymine glycol. Eight-oxo-dG [6] is generated when the C8-OH adduct radical, produced by the reaction between the highly reactive hydroxyl radical and guanine, loses an electron and a proton. The C8-OH adduct radical may also be reduced by uptake of an electron and a proton forming 7-hydroxy-8-hydroxyguanine which is subsequently converted to 2,6-diamino-4-hydroxy-5-formamidopyrimidine, a second major oxidation product of guanine. Mutations that may arise due to the formation of 8-oxoG involve GC → TA transversions [7].

Specialized repair proteins continuously scan the genome. When they encounter a lesion such as an AP site, a mismatched base or a structurally altered base, they start one of the DNA repair mechanisms that ultimately lead the restoration of the genetic information. DNA repair is carried out through several different pathways that process different types of DNA damage; these include BER, NER, homologous recombination, non-homologous end joining, and mismatch repair. When a lesion cannot be repaired, the only resource that allows cell survival is lesion bypass, operated by a process of trans-lesion synthesis

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in which a polymerase or replicative assembly encounters a non-coding or miscoding lesion, inserts an incorrect nucleotide opposite to the lesion and then continues elongation [8]. Eventually, DNA damage activates checkpoint regulators such as p53, SMC1, FANCD2, BRCA1 that promote cell cycle arrest and apoptosis. The impairment of any component of this machinery increases dramatically mutagenesis and promotes cancer. Indeed, clearance of damaged cells by apoptosis is considered the last barrier against the fixation of excessive deleterious mutations.

However, the role *in vivo* of ROS levels and apoptosis on mutagenesis has not been clearly defined.

Endogenous ROS Functions

ROS are highly reactive species that can exert mutagenic effects on both mitochondrial and nuclear DNA. Moreover, ROS can alter lipids and proteins. Hydroxyl radical produced by the reaction of Fe^{2+} and H_2O_2 , for example, inactivates the Krebs cycle enzyme aconitase. In turn, it can contribute to further ROS generation which amplifies the damage. Oxidized proteins are recognized by proteases and degraded; new replacing proteins are synthesized *de novo*.

Specific enzymes such as superoxide dismutases (Mn-SOD), catalases (CAT), lactoperoxidases, glutathione peroxidases (Gpx) and peroxiredoxins (Prx) scavenge ROS levels to prevent the harmful consequences (oxidative stress) of their accumulation. The presence of small antioxidant molecules such as ascorbic acid, tocopherol, uric acid, and glutathione also helps to scavenge free radicals. Vitamin E, for example, can break the covalent links formed by ROS between fatty acid side chains in membrane lipids [9].

Superoxide dismutases represent the most important class of enzymes that defend cells from oxygen exposure. In mammals, three forms of superoxide dismutase have been described: SOD1 is located in the cytoplasm, SOD2 in the mitochondria and SOD3 in the extracellular space. They catalyze the dismutation of superoxide into O_2 and H_2O_2 . Mice deficient for SOD have a decreased life span and an elevated incidence of liver cancer [10]. H_2O_2 is the most stable ROS; it can diffuse from mitochondria into the cytosol, where it is further metabolized by catalases, Gpx1 and PrxIII. Catalase is concentrated in peroxisomes located next to mitochondria and reacts with the H_2O_2 to catalyze the formation of H_2O and O_2 . Mice lacking catalase exhibit a retarded rate in decomposing extracellular H_2O_2 with respect to wild type and a severe brain injury following physical trauma [11]. Notably, mice that overexpress mitochondrial catalase show an extended lifespan [12]. Glutathione peroxidase reduces H_2O_2 by transferring the energy of the reactive peroxides to a very short sulfur peptide called glutathione. Peroxiredoxins degrade H_2O_2 in the mitochondria, cytosol and nucleus compartments.

Regardless their damaging effect, ROS play important functions in the cell. They are essential for the defense of the organism against pathogens and they also play a major role in the regulation of intracellular signaling pathways [13].

Neutrophils kill engulfed pathogens by using myeloperoxidase, an enzyme that catalyzes the reaction of H_2O_2 with Cl^- to produce the strongly antiseptic ClO^- ion. In addition, neutrophils produce ROS by NADPH oxidase. This enzyme is also present in macrophages where localizes in the lysosome membrane and catalyzes the synthesis of superoxide anion to destroy the phagocitated host.

Moreover, NADPH oxidase has been found in cells, such as

fibroblasts, endothelial cells, osteoclasts and chondrocytes [14] where they have no role in host defense. The rate of ROS generation by NADPH oxidase in these cells is lower compared with neutrophils. This suggests, together with the different localization of the enzyme, that ROS, in these cells, are involved in signal transduction pathways [15]. Indeed, ROS are implicated in cell growth and differentiation, both activating and inhibiting apoptosis, and, at higher concentrations, inducing necrosis [16].

DNA microarray analysis have shown that H_2O_2 regulates the expression of almost 100 genes [15] and the activation of transcription factors such as NF- κ B [17] and AP-1 [18], Myb and Ets [19]. Moreover, several enzymes involved in cell signaling are regulated by H_2O_2 ; for example, H_2O_2 produced in response to stimulation with Epidermal Growth Factor can cause reversible inactivation of Protein Tyrosine Phosphatase 1B [20] and PTEN [21] by oxidizing its catalytic site cysteine.

H_2O_2 does also regulate the opening of the mitochondrial permeability transition pore (MPTP), which plays a key role in the intrinsic apoptotic pathway. Indeed, opening of the MPTP alters mitochondrial membrane permeability and allows solutes to diffuse from mitochondria to cytoplasm, in particular apoptogenic factors, such as cytochrome *c* that once released in the cytosol triggers the apoptotic cascade.

P66Shc Regulates Mitochondrial ROS Production and Apoptosis

P66Shc is one of the 3 isoforms encoded by the human and mouse ShcA locus. ShcA was identified in 1992 by low-stringency hybridization to human cDNA libraries, using a SH2-coding sequence as a probe [22]. It is located on chromosome 1q21. The originally isolated shcA transcript displayed two in-frame ATGs and was shown to encode two ubiquitously expressed polypeptides: p52Shc and p46Shc.

Members of the Shc family are characterized by the PTB-CH1-SH2 modularity where: CH1 (collagen homologues 1) is a glycine/proline rich region, containing 2 major phosphorylation sites: Y239-240 and Y317; SH2 (Src homologous type 2) is the C-terminal domain involved in protein-protein interaction and mediates the formation of multi-protein signaling complexes; PTB (phosphotyrosine binding domain) is also able to bind non-phosphorylated Tyr-containing peptides. P66Shc contains an additional amino-terminal proline rich region, named CH2.

Isoforms p46Shc and p52Shc serve as phosphotyrosine adaptor molecules in various receptor-mediated signaling pathways. Once phosphorylated, they recruit the GRB2/SOS complex to the plasma membrane [23] and subsequently activate the mitogenic-activated protein kinase (MAPK) cascade. P52Shc and p46Shc function as initiators of the Ras signaling cascade in various non-neuronal systems [24]. P66Shc is a target of receptor tyrosine kinases [25] and it is able to bind the Grb2/SOS complex. However, p66Shc is not involved in Ras signaling regulation and its overexpression has a negative effect on the RAS-MAPK-*fos* pathway in response to EGF. On the contrary p66Shc converts intracellular oxidative signals into apoptosis [26].

A fraction of p66Shc has been observed within the mitochondrial inter-membrane space (IMS). However Import mechanism of p66Shc into mitochondrial IMS has not been clearly identified. During apoptosis levels of p66Shc in mitochondria increase. Some stress kinases such as Jnk-1 and Pkc-B phosphorylate p66Shc in Ser36, and Pin-1 induces its proline-isomerization [27]; these post-translational modifications

are involved in p66Shc translocation within mitochondria. In basal conditions, mitochondrial p66Shc associates to a high molecular weight complex of about 670 kDa and to the mitochondrial chaperon mtHsp70 [28] and components of TIMTOM import complex [29]. Notably, treatment of cells with pro-apoptotic stimuli such as UVC or H₂O₂ induces the dissociation of these complexes and the release of monomeric p66Shc, which then reacts with cytochrome c [28] sequestering electrons from the ETC to generate H₂O₂ [30,31].

Deletion of p66Shc gene in mice results in increased resistance to oxidative stress [26] and reduced body weight (due to reduced fat accumulation) [32]. Interestingly these mice have an approximately 30% extension of life span. Notably, p66Shc deletion in mice improves resistance to hyperglycemic damage in a diabetic model of nephropathy as well as to cardiovascular disease, due the reduction of apoptosis and cell loss [33,34].

P66Shc knock out (p66Shc^{-/-}) fibroblasts are resistant to apoptosis induced by many signals, relating to the increased H₂O₂-sensitivity conferred by over expression of p66Shc [26]. Being the production of energy mainly associated with glycolysis instead of ETC [35], p66Shc^{-/-} cells show a reduced mitochondrial metabolism.

P66Shc is not only involved in ROS generation, it regulates the expression of ROS scavenging enzymes through inhibition of FOXO transcription factors [36]. Indeed, in adipocytes, p66Shc is a downstream target of the insulin signaling pathway [31]. Activation of p66Shc leads to the activation of AKT by the direct effect of p66Shc-generated H₂O₂ on PTEN. The effect of p66Shc is more marked on FOXO, a critical transcription factor downstream of AKT [31,37].

In animal tissues, the extent of oxidative damage correlates with the levels of expression of p66Shc. Indeed, oxidative damage is higher in the lung, spleen, liver, and skin where p66Shc is expressed at high levels, whereas no significant oxidative damage is found in brain where p66Shc is expressed at low levels [38]. Notably, the amount of 8-oxo-dG in nuclear and mitochondrial DNA was found to be lower in p66Shc^{-/-} mice.

Mutation Assays *In Vivo*: Biological Systems

Several approaches can be used to study mutation accumulation *in vivo*. In the past, it was only possible to detect chromosome rearrangements via cytogenetic analysis on proliferating cells, such as lymphocytes. Later, also point mutations could be detected in such cells, using selectable marker genes. The most popular was the X-chromosomal gene for hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus test [39].

This enzyme converts purines (hypoxanthine, guanine, or 6-mercaptopurine) to the corresponding nucleoside monophosphates. Accumulation of these molecules in cells has cytotoxic effects. The assay is based on the fact that if mutations occur in the Hprt locus the function of the HPRT enzyme is compromised and cells are able to survive treatment with purine analogues.

This method was largely used to study accumulation of mutations with age in lymphocytes from humans and mice. The problem related to this assay is that it poorly reflects the *in vivo* system. The analysis is limited to active proliferating cells whereas the majority of adult cells only rarely undergo cell division.

With the advent of transgenic mouse technology, a completely different approach became possible. The use of reporter vectors integrated in mouse chromosomes allows studying mutations

accumulation in every rodent organ. Reporter vectors are plasmids or phage packaging containing prokaryotic genes that can be easily recovered in host bacteria (mutated in the gene used as reporter) [40].

Vectors appear to be transcriptionally inactive as indicated by heavy methylation of CpG sites and absence of mRNA [41,42]; therefore mutations of the transgene are neutral.

The spontaneous mutation frequency in transgenic portions of the genome appears to be somehow higher than in endogenous genome, however some comparisons indicate that transgenes and endogenous genes have essentially the same reaction upon treatment with mutagens [43]. Endogenous genes are likely to differ substantially respect to mutation susceptibility and spectrum, due to variations like size, genomic location and function in a given organ or tissue. They are transcriptionally active and thus are subject to transcription-coupled DNA repair (TCR). It is unlikely that adducts in the transgene would be subject to TCR, they would rather be processed in a much slower timeframe via GGR [44].

Unrepaired DNA lesions that persist through DNA replication are clearly of most concern in tissues with a rapid turnover. In post-mitotic organs, mutations may be fixed by DNA repair process: for some types of repair, such as repair of double strand breaks, this may be significant.

Several types of shuttle vector-containing transgenic mouse models are now available. The first was described by Gossen and Vijg [45] and uses bacteriophage λ . The transgenic model was originally constructed by microinjection of the bacteriophage λ gt10-*lacZ* shuttle vector into fertilized CD2 mouse oocytes. *lacZ* is part of *lac* operon, required for the transport and metabolism of lactose in bacteria. *lacZ* encodes for the enzyme β -galactosidase that catalyzes the hydrolysis of lactose into glucose and galactose. Rescue of the vectors is performed by exposing total genomic DNA, isolated from different organs or tissues, to an *E. coli* *in vitro* packaging extract. In order to avoid host restriction, both the packaging extract and the plating cells need to be derived from a completely host restriction negative *E. coli* strain, such as *E. coli* C [46]. The phages obtained are plated on a Δ lacZ *E. coli* strain for the selection of mutant and non-mutant *lacZ* phages.

Another transgenic model, based on the *lac* operon, was developed by Kohler to measure DNA mutation rate [47]. The mutational target is *lacI* instead of *lacZ*. *LacI* protein negatively regulates *lacZ* expression; it acts by binding the operator sequence upstream of the *lacZ* gene when lactose is absent. Consequently, in this system, infection of *lacZ*+*E. coli* host cells allows the selection of phages contain a mutant or non-mutant *lacI* gene.

Both the *lacZ* and the *lacI* bacteriophage lambda systems have now been extensively used for studying mutation accumulation *in vivo* for genetic toxicological analysis. There are other systems based on bacteriophage λ : the cII selection [48]. Glazer et al. developed transgenic mice equipped with a λ supF shuttle vector [49] and Malling and Burkhart generated the phiX174 am3 cs70 transgenic mouse system [50].

In addition to λ based models, mouse carrying plasmid vectors have been generated. The main differences between these models consist in the size of the shuttle vector and in how it is rescued. Unlike the phage based system, in which very high molecular weight genomic DNA must be isolated in order to obtain intact vectors with high efficiency, plasmids can be isolated from genomic DNA with a very high efficiency since multi-copy concatemers are integrated in mouse genome.

With the λ system, it is impossible to detect deletions and insertions

larger than 5 kb because in vitro packaging requires λ vector with a minimum size of 42 kb and a maximum size of 52 kb. The plasmid model, on the contrary, allows to identify deletions arising within the concat3mer as well as deletions extending from a lacZ target gene into flanking chromosomal sequences that can be recovered, detected and characterized [51].

Several lacZ transgenic lines were derived by microinjection and bred to homozygosity. In our experiments, we used C57BL/6J line 30 that harbors 21 copies of pUR288 tandemly integrated on chromosome 11. Line 60 harbors a total of about 20 copies of the plasmid integrated in a head-to-tail fashion at two chromosomal sites: chromosome 3 and chromosome 4. It was demonstrated that both lines give comparable results in mutagenesis studies [52]. Other plasmid transgenic mouse models were generated: rpsL transgenic mouse [53] (and 6-TG selection assay [54]).

LacZ-plasmid mice have been extensively characterized, and mutation accumulation over time in several organs has been described. At young age, mutation spectra rates are similar, whereas over time small intestine, characterized by a very high cell proliferative activity, undergoes the largest age-related increase in mutation rate. In liver and heart, mutant frequencies increase with age from birth to 34 months; in brain, testes and spleen an increase is observed only between birth and 4-6 months [2]. Also the types of mutations accumulated vary from organ to organ: for example in small intestine the age-related increase is entirely due of point mutations, while in liver both point mutations and genome rearrangements occur.

Mitochondrial Ros and Apoptosis Effect on LacZ Mutation Rate: The Lesson from P66shc

We measured the accumulation of mutations in p66Shc^{-/-} mice using the LacZ reporter transgenic mouse model. Double heterozygous (LacZ^{+/-} -p66Shc^{+/-}) first generation mice (F1) were crossed in order to obtain LacZ^{+/+}-p66Shc^{-/-} animals. The genotyping strategy used to assess the presence of the LacZ transgene does not to discriminate between LacZ^{+/+} and LacZ^{+/-} animals. Therefore, F2 mice that were p66Shc^{-/-} were crossed with a WT C57/Bl6 mouse and genotype of the progeny was used to determine the genotype of the F2 mouse. Indeed, all mice of the progeny are LacZ^{+/-} when a LacZ^{+/+} animal is crossed with a WT mouse. F2 LacZ^{+/-} mice, on the contrary, generate both, LacZ^{-/-} and LacZ^{+/-} progeny. F2 LacZ^{+/+} p66Shc^{-/-} mice were crossed to establish the colony.

LacZ Plasmids were integrated head to tail in the mouse genome. To determine mutation frequency, lacZ transgenes were rescued from the genomic DNA by excision with HindIII, followed by purification using magnetic beads, pre-coated with LacI repressor protein. Plasmids were then ligated and transferred to *E. coli* C (Δ lacZ, *GalE*) using electro-transformation. 1:1000 of transformants was plated in medium with X-gal to determine the total number of plasmids rescued. The remainder was plated in the presence of high concentrations of the lactose analogue p-gal that only allowed cells harboring a mutant lacZ gene to grow. Mutants were characterized using restriction analysis. Results revealed [55] that size change mutations rather than no change mutations accumulated particularly in MEFs and small intestine from p66Shc^{-/-} mice.

ROS induce DNA adducts and breaks that activate the genome damage response to induce repair, cell cycle arrest and eventually clear the damaged cell.

Indeed, studies on p66Shc^{-/-} mice as other transgenic animals have

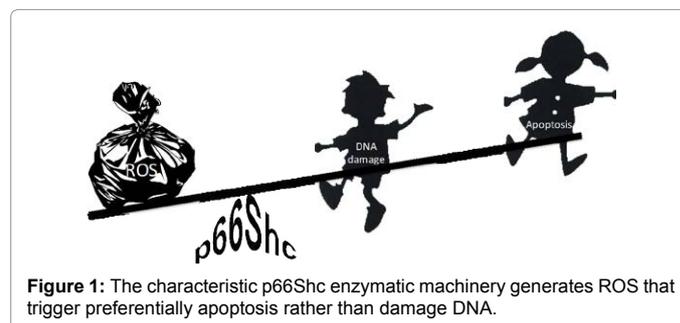


Figure 1: The characteristic p66Shc enzymatic machinery generates ROS that trigger preferentially apoptosis rather than damage DNA.

shown that intracellular levels of ROS negatively correlate with stress resistance and life span [26]

We have found that the overall LacZ mutation frequency was unaffected by the deletion of p66Shc indicating that the amount of ROS generated by p66Shc, although relevant to trigger mitochondrial apoptosis, are not genotoxic. Indeed, p66Shc^{-/-} mice showed normal tumor incidence.

However, the deletion of p66Shc changes the type of mutations that are accumulated over time and upon stress, as size change mutations were significantly higher in LacZ p66Shc^{-/-} than in LacZ background, both in the small intestine from old and young irradiated mice and stressed MEFs.

Interestingly, LacZ and p66Shc^{-/-} MEFs treated with H₂O₂ show similar frequency of size change mutations. Different size change frequency of MEFs treated with H₂O₂ suggests that susceptibility to die of the p66Shc^{-/-} cells is independent to DNA damage. In agreement with this hypothesis we have observed that the deletion of cyclophilin D (CypD), that induces apoptosis through the opening of mitochondrial permeability transition pore with the same mechanism of p66Shc, increases size change mutations in the small intestine of old mice [55].

P66Shc and CypD are expressed in the liver and the fact that the small intestine but not the liver showed differences in the mutation rate compared to the WT, suggests that tissues with high turnover particularly feel the effect of p66Shc and CypD, thus supporting the hypothesis that impaired apoptosis plays a role in mutation rate.

Finally, p66Shc deletion protects from stress and degenerative diseases but may affect the integrity of some tissues allowing the accumulation of particularly mutated cells (Figure 1).

To what extent these cells guarantee tissue function is questionable. Recently, we have found that p66Shc deletion is counter-selected when mice are maintained in harsh conditions (open field under cold and competition for food) that mimic wild, indicating that p66Shc is essential for fitness under stressing natural conditions but redundant in protected environment [56]. So, p66Shc^{-/-} mice can survive to accumulate a peculiar spectrum of mutations only in laboratory conditions.

In conclusion, the particular spectrum of LacZ mutations in p66Shc^{-/-} underlines the importance of cell death rather than the redox induced mutagenesis and suggests that specific genetic sets, in precise environments, determine somatic mutation rate.

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