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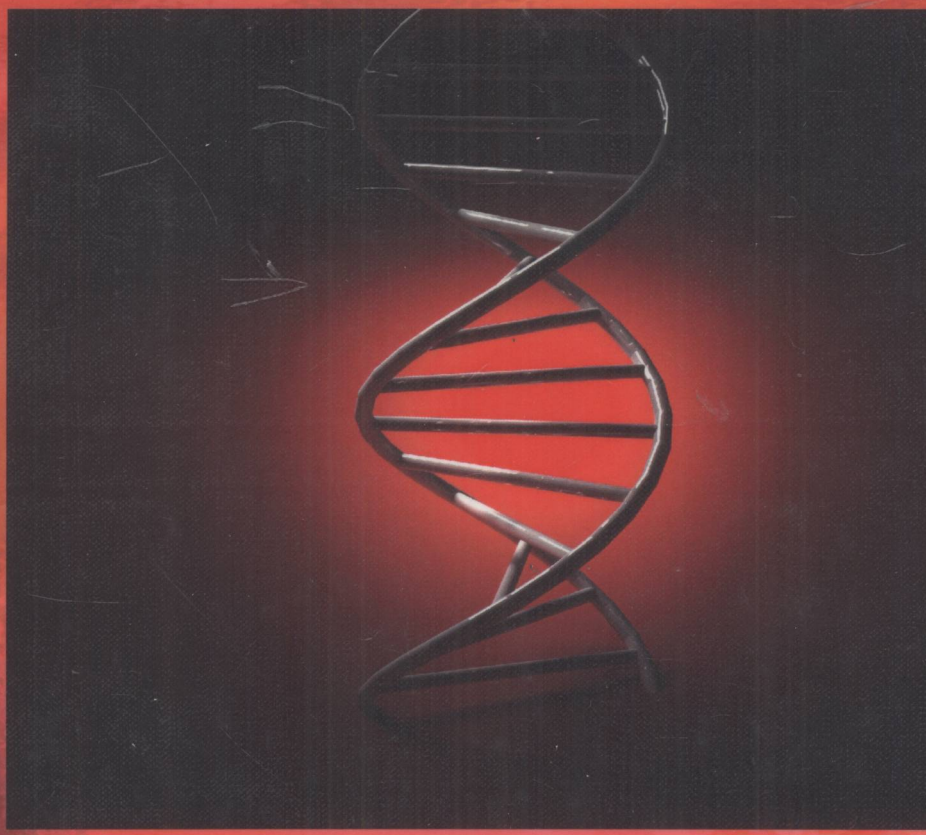
Progress in DNA Damage Research



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PROGRESS IN DNA DAMAGE RESEARCH

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PROGRESS IN DNA DAMAGE RESEARCH

Preface

This new book presents the latest research on DNA damage, which due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. While this constitutes only 0.000165% of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as tumor suppressor genes) can impede a cell's ability to carry out its function and appreciably increase the likelihood of tumor formation.

The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified. These modifications can in turn disrupt the molecules' regular helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. Unlike proteins and RNA, DNA usually lacks tertiary structure and therefore damage or disturbance does not occur at that level. DNA is, however, supercoiled and wound around "packaging" proteins called histones (in eukaryotes), and both superstructures are vulnerable to the effects of DNA damage.

Chapter I - Somatic hypermutation (SHM) in the immunoglobulin (Ig) variable (V) region and a few other genes in B lymphocytes is a unique process that mainly occurs in germinal centers (GCs). The process is essential for the immune defense, but it has been implied to be tumorigenesis. Here I review progress made in the past decades in unraveling the molecular basis of SHM. SHM was first visualized in the Ig protein and then detected at the DNA level. It starts 100 – 200 bp downstream of the promoter, reaches a peak in the V region and is gradually diminished after the peak. Transcription is generally thought to be required for the SHM process. Although the Ig enhancers are crucial for SHM in the transgenic mouse model, the Ig promoter or the V region sequence can be replaced with a non-Ig one. The preferences of C,G transitions versus transversions, A versus T mutations, and mutations at hotspots indicate that the mutation process is not random, suggesting that there is a mutator factor responsible for the mutations. The mutator factor is activation-induced cytidine deaminase (AID). The enzyme is a single-stranded DNA deaminase and is able to directly attack DNA using tagged AID proteins purified either from insect cells or from bacteria. It preferentially targets WRC hotspots. Moreover, AID acts on supercoiled DNA and has deamination activity on both DNA strands. In vivo, the AID protein is phosphorylated and associated with RPA in order to efficiently deaminate cytidine during transcription. Several major DNA repair pathways have been screened for a possible role in

the mutation process. Among these, nuclear excision repair (NER) is apparently unrelated to SHM, while mismatch repair (MMR) and base excision repair (BER) are involved in the mutation process by expanding the C/G>T/A mutation spectrum caused by AID to C,G,A,T transitions/transversions with the help of error-prone DNA polymerases (pols). Typically, pol η generates A,T mutations during MMR. Although much has been learned regarding the AID function, how AID targets DNA during transcription and why it only attacks Ig and a few other genes remain relatively unknown. Two SHM models are discussed in this review.

Chapter II - Chromosomal aberrations (CA) play a major role in genomic instability, cancer development, birth defects and infertility. DNA double strand breaks (DSB) are the main lesions leading to the formation of CA. DSB can be induced by clastogenic agents either directly (e.g. ionizing radiation, restriction endonucleases) or indirectly (e.g. UV radiation, most chemical compounds) or even arise spontaneously through cellular processes (e.g. DNA replication, DNA excision repair, transposition, mitotic recombination or endogenous oxidative damage). The formation of CA can be modulated by structural and functional nuclear factors such as the nature of DNA sequences, chromatin conformation, cell cycle stage and efficiency of DNA repair mechanisms. The nucleotide sequence could influence the induction of DNA damage in relation to their differential susceptibility or the toposelectivity for the production of lesions due to DNA conformation. Chromatin status and epigenetic modifications during cell cycle progression influence the frequency and distribution of intra- or inter-chromosomal damage. Highly packed chromatin could protect DNA more efficiently than loose chromatin. Thus, less condensed and hyperacetylated chromatin within trascriptionally active (poised) regions could be more damage-prone. Besides, chromatin regions remodelled during DNA replication (S phase) or along chromosome condensation in G2/M transition could also be more susceptible to DNA-damaging agents. Additionally, DNA replication influences the frequency and distribution of CA since DNA lesions different from DSB could be converted into DSB during this process. The processing of DNA lesions by different repair mechanisms may also induce CA. Homologous recombination (HR), the most accurate DSB repair process, requires sequence homology for DNA repair and hence may lead to CA if recombination involves homologous sequences in different chromosomes (ectopic HR repair). Chromosome territorial arrangement in the interphase nucleus where active chromatin loops spatially intermingle between neighbouring chromosome domains could facilitate ectopic HR repair within these regions.

Chapter III - In addition to all the benefits pesticide usage can bring to crop production and normal daylife, these compounds also present several harmful properties if not correctly used. Their non-specific action increases the possibility of all living organisms (including man) exposed to pesticides undergo severe acute or chronic poisonings.

Pesticides are recognized genotoxic compounds that can induce direct and indirect damage in DNA. They are therefore capable of damaging directly the bases that constitute the DNA molecule (resulting in direct DNA breakage, replication on a damaged DNA template, inhibition of DNA synthesis and other mechanisms) but are also associated with the production of free radicals that can also change the structure of DNA.

Traditionally, genetic damage was observed by means of chromosomal aberrations. However in the more recent decades new and improved techniques were developed and proved to be useful in understanding the potential of these compounds to induce DNA damage. Nevertheless the validation of these biomarkers is still an extremely important issue that can not be overlooked.

Data obtained over the years indicate that information given by biomarkers of effect should not be single-handedly but instead analyzed together with biomarkers of exposure and susceptibility. Integrated data provides much more useful data that can aid to the prediction, cause, diagnosis, progression, regression, or treatment of possible disease.

Lastly and since genetic damage can be on the basis of chromosomal instability, the key event leading to the first stage of carcinogenesis, this subject will also be approached. The occurrence of such phenomena may play a crucial role in cancer incidence among pesticide exposed populations and several authors have already reported an increased number of cases of different types of cancer in farm populations.

Chapter IV - Oil spills are one of the most frequent ecological disasters giving rise to the pollution of enormous coastal areas and affecting also the local flora and fauna. As a direct consequence of them, a large number of individuals is always involved, as they take part in the different tasks derived from the need of cleaning the spilled oil and recovering the natural environments. From a toxicological point of view, oil is a complex mixture of compounds that can penetrate into the body burden through dermal, respiratory or digestive routes. Despite the huge number of spills occurred all around the world, the international literature dealing with the harmful effects of this exposure is very scarce and restricted to the acute and psychological effects. This seems paradoxical attending to the large number of carcinogenic agents composing this mixture.

So, after the *Prestige* oil spill (November 2002, NW of Spain) and taking into account the seriousness of the catastrophe, the large number of individuals involved, the damaging character of oil components and especially the lack in the scientific literature of reports considering the consequences of this exposure from a genotoxic or carcinogenic point of view, we developed an extensive biomonitoring study including effect and susceptibility biomarkers. Three groups of exposed individuals whose exposure during the recovery of *Prestige* oil polluted areas differed quantitatively (short and acute or more prolonged in time) and qualitatively (as a consequence of the different methods used for this purpose) were included. Environmental concentrations of volatile organic compounds were evaluated by means of passive dosimeters in each exposure group. Two types of effect biomarkers were applied: the comet assay, characterized by its high sensitivity in population studies and for reflecting also DNA repair phenomena, and two well established cytogenetic assays, micronucleus test and sister chromatid exchanges. Moreover, due to the fact that individual differences in terms of susceptibility to xenobiotics have been extensively reported and mainly attributed to some polymorphisms in genes encoding for biotransformation enzymes and DNA repair proteins with functional consequences, a complete set of the most relevant were also included in this study.

Chapter V - Nucleotide excision repair (NER) is one of the most important DNA repair systems, and certainly the most versatile: it can handle UV-induced lesions, bulky chemical adducts, protein-DNA adducts, intra-strand crosslinks, and it participates in the repair of

inter-strand crosslinks. Hereditary deficiencies in NER result in several dreadful diseases, such as the highly cancer-prone xeroderma pigmentosum, and Cockayne syndrome, which is characterized by severe developmental and neurological defects.

Yet, despite its obvious importance, NER is strongly attenuated in many cell types; mostly in differentiated cells (neurons, macrophages, muscle fibres, etc.), but also in intermittent mitotic cells (e.g. B lymphocytes) and even in quiescent stem cells. A teleological explanation for this attenuation is that cells that do not divide may dispense with the burden of repairing the bulk of their genome, since they do not replicate it. This parsimonious strategy, however, demands that transcribed genes are still proficiently repaired for the cell to function normally. This is achieved by two subpathways of NER: transcription-coupled repair (TCR) and transcription domain-associated repair (DAR). DAR operates on both strands of active genes, whereas TCR enhances the repair rate of the transcribed strand by using the translocating RNA polymerase II as a lesion sensor.

Our recent work has shed some light on the mechanisms underlying NER attenuation in differentiated cells. We found that the ubiquitin-activating enzyme E1 is able to restore proficient NER in extracts from differentiated macrophages, suggesting that an NER enzyme must be activated by ubiquitination. This activation does not occur in differentiated cells, most likely because of a decrease in phosphorylation of the E1 enzyme. The function of E1 is to activate ubiquitin and transfer it to a battery of E2 enzymes. It is likely that some of these E2s, such as the one used by NER, are unable to interact with the hypo-phosphorylated form of E1 that we have observed in macrophages.

The molecular details of TCR are still elusive, aside from the fact that a stalled RNA polymerase II recruits NER enzyme to the site of damage. By contrast, we have evidence that DAR is simply a concentration of NER enzymes in the nuclear sub-compartments in which transcription takes place. The remaining repair-proficient NER enzymes likely accumulate in these transcription factories, accounting for the persistence of NER in active genes, despite its attenuation at the global genomic level.

Chapter VI - The cellular response to genotoxic stress that damages DNA includes cell cycle arrest, activation of DNA repair, and in the event of irreparable damage, induction of apoptosis. However, the signals that determine cell fate, that is, survival or apoptosis, are largely unknown. Recent studies have shown that several pro-apoptotic kinases undergo nuclear-cytoplasmic shuttling in response to DNA damage. Importantly, whereas precise mechanisms for the shuttling remain uncertain, this mechanism has consequences for induction of apoptosis and implies that proper localization is central to the function of pro-apoptotic kinases. This progress review highlights recent efforts demonstrating that the nuclear targeting of kinases might be a novel and essential regulatory mechanisms that directly influences the induction of apoptosis.

Chapter VII - Oxidative stress, a biochemical condition that is characterized by an imbalance between the presence of relatively high levels of toxic reactive species and antioxidative defense mechanisms, has been linked to various cellular philology and disorders characterized by cell death, such as stem cells of neural and hematopoietic systems. Recent studies indicate that common chromosome fragile sites, non-random targets of double stranded breaks under replication perturbation that are characteristic of tumors, paradoxically encode the tumor suppressor FHIT gene, which modulates response to oxidative stress and

DNA damage. Since it is inactivated in precancer or early stages of carcinogenesis, we propose that the FHIT gene plays a role in the creation of cancer initiating cells. Mechanistic roles of FHIT in modulating oxidative stress and DNA damage checkpoints of cancer initiating cells are also discussed.

Chapter VIII - It is generally accepted that DNA damage checkpoint is the mechanism that allows time for DNA damage repair. However, several lines of evidence challenge this paradigm, especially, in the case of G1 checkpoint. The first evidence is the complete difference between the repair kinetics of DNA double-strand breaks (very rapid) and the timing of G1 checkpoint induction (very slow) after ionizing radiation. The second evidence is that inactivation of p53, which is a central player of G1 checkpoint, does not render cells radiosensitive, rather, such cells become radioresistant. Moreover, it was shown that G1 arrest persists almost permanently after irradiation, until the time when most of the initial damage should be repaired and disappear. Therefore, cells should have a mechanism to maintain G1 checkpoint signaling by amplifying the signal from a limited number of damage. In this review, we discuss what is the *bona fide* role of G1 arrest and how G1 checkpoint signal is maintained long after irradiation.

Chapter IX - Severity of ischemia/reperfusion injury accompanied with generation of reactive oxygen species is reduced by hypoxic preconditioning, although the precise mechanism is not completely understood. Recently, several authors suggested that mitochondrion-mediated apoptosis and the pathway via hypoxia-inducible factor-1α (HIF-1α) contributed to the mechanism. In this study, we determined mitochondrial morphology, mitochondrial DNA (mtDNA) damage, and the growth in HEK293 cells acutely exposed to H_2O_2 as cells were pretreated with cobalt to mimic hypoxic preconditioning. Mitochondrial swelling and mtDNA damage were observed in a manner dependent on H_2O_2 concentration. When 0.4 mM H_2O_2 was exposed to cells where HIF-1α expression was induced with 0.1 mM $CoCl_2$, mtDNA damage was suppressed. When the amount of H_2O_2 remaining in the medium was determined, the rate constant for H_2O_2 depletion by the cobalt-treated cells tended to be faster than that by no treated cells. When cells were exposed to H_2O_2, number of cells 4 days later decreased to 3.7% of that without exposure. However, number of cells pretreated with $CoCl_2$ (15.7 % of that without exposure) was significantly greater than that without cobalt treatment. This is consistent with the fact that heme oxygenase of antioxidant enzyme is a target gene of HIF-1α. These results suggest that cobalt-induced HIF-1α activity would ameliorate the suppression of cell growth through protecting mtDNA damage caused by H_2O_2.

Chapter X - Electromagnetic waves containing γ– (X-) rays and light in the ultraviolet (UV) and visible (VIS) ranges have direct and indirect effects on DNA. γ– (X-) radiation generates double-stranded breaks, and UV-C/UV-B radiation generates pyrimidine dimers. Such DNA damage can be lethal. UV-A/VIS radiation in the presence of photosensitizers oxidizes guanine, but oxidative lesions of guanine cause point mutations that are less lethal than double-stranded breaks and pyrimidine dimers, and thus generally do not lead to cell death. In particular, photosensitizing frequently causes G–C to T–A and G–C to C–G transversions. 8-Oxoguanine (8-oxoG) causes G–C to T–A transversions by formation of an 8-oxoG-A pair. However, 8-oxoG does not cause G–C to C–G transversions since guanine is not incorporated opposite 8-oxoG. Recently, several oxidative lesions of guanine have been

reported as candidates that cause G–C to C–G transversions. Our discussion with respect to the A-rule suggests the possibility that the base pairing between guanine and the oxidative lesion of guanine involves hydrogen bonding. In addition, telomere cleavage at the site of oxidative lesions of guanine may contribute to cell senescence.

Chapter XI - It is well established that sperm quality plays a crucial role in human reproduction. Increasing number of studies concerning DNA fragmentation analysis of human semen and testicular cells suggest that this can be a parameter of semen quality, because sperm chromatin abnormalities or DNA damage may result in male infertility. Sperm DNA integrity is essential for the accurate transmission of genetic information and is of vital importance in the initiation and maintenance of a pregnancy in vivo and in vitro. The DNA fragmentation in human sperm has been related to unexplained infertility. Moreover, the causes of DNA fragmentation in ejaculated human sperm are unclear, and several mechanisms have been proposed, like defects in the chromatin compaction during spermiogenesis, reactive oxygen species production by immature spermatozoa, apoptosis during spermatogenesis and exposure to environmental toxins. Also, there are a variety of methods to study sperm genetic integrity that will be discussed in this review, as well as the implication of the DNA fragmentation study on assisted reproduction results.

Chapter XII - Massive damage of human sperm DNA has focused great attention in the era of assisted reproductive techniques (ARTs). Indeed, the use of procedures that bypass the natural barriers to fertilization may increase the risk that a sperm with damaged DNA fertilizes an oocyte, particularly in view of the increasing evidence that sperm DNA damage is associated to male infertility.

Massive sperm DNA damage consists in the presence of modified bases, of single and double stranded DNA breaks and of defects in sperm chromatin maturation. Most studies focused on single and double DNA breaks (sperm DNA fragmentation) and most clinical investigations have used sperm DNA fragmentation as an index of sperm DNA damage in studies on the impact of such a damage on the ARTs outcome.

Despite the extensive efforts in this field, the origin and the mechanisms responsible for sperm DNA fragmentation are not yet clarified, although it appears clear that their knowledge might give new inputs in the treatment of male infertility. In addition, reported investigations on the clinical meaning of sperm DNA integrity are conflicting and whether sperm DNA fragmentation may represent a new useful parameter to predict male fertility potential and/or to counsel couples involved in ARTs protocols is still controversial.

Beside sperm DNA fragmentation, evidence is accumulating, in recent years, on defects in chromatin protamination as well as on their impact on male fertility.

In this chapter the authors discuss the origin of DNA fragmentation and of impairment of protamine deposition in chromatin as well as their consequence for male fertility potential and reproduction.

Chapter XIII - Sperm DNA damage appears to be one of the main causes of decreased reproductive ability of men, in natural as well as in assisted reproduction. The objective of this chapter is to inquire into our present knowledge of sperm DNA fragmentation, with special reference to the biological meaning and clinical importance of sperm DNA damage in assisted reproduction.

In particular, thw authors analyzed three main aspects:

- The relationship between sperm DNA damage and reproductive outcome: this part focuses on the available literature in the attempt to assess the potential impact of sperm DNA fragmentation on the outcome of assisted reproduction techniques (ARTs).
- Strategies to reduce sperm DNA damage: we analyzed the various strategies and treatments currently adopted to handle the problem of DNA fragmentation and decrease its incidence in assisted fertilization.
- Sperm cryopreservation and DNA damage: the authors assessed the influence of cryopreservation on sperm DNA and ART outcome.

Chapter XIV - As the sperm cell's primary role is to transmit a haploid genome to the oocyte, it is essential that the integrity of this genome be evaluated as part of the standard semen analysis. The Sperm Chromatin Dispersion (SCD) test is a simple and easy to implement laboratory procedure used to determine the frequency of sperm cells with fragmented DNA. While there appears to be a strong relationship between fertility (fertilization rate, embryo quality and pregnancy rate) and the frequency of spermatozoa with fragmented DNA, there is nevertheless, some controversy over the specifics of these correlations as they relate to pregnancy. These disparities may be due to the complex interactions between the presence of different types of quantitative and qualitative DNA damage in the sperm cell and the competence of the different DNA repair pathways provided by the oocyte. The importance of sperm DNA assessment as a routine technique for the clinical andrologists is emphasized by the positive relationship of sperm DNA fragmentation with physical abnormalities, such as varicocele, venereal pathogens and neoplasia.

Chapter XV - Over the past decade the relatively new Fast Micromethod® for detecting DNA damage (strand breaks, alkali-labile sites and incomplete excision repair) and related DNA integrity in cell suspensions or tissue homogenates in single microplates has been developed. The technique is based on the ability of the specific fluorophore PicoGreen® to preferentially interact with high integrity DNA molecules, dsDNA, allowing direct fluorimetric measurement of fluorophore-dsDNA complex decay in alkaline conditions. This analytical technique allows simple and fast simultaneous analysis of DNA integrity in numerous, low DNA content samples. Research has confirmed that the Fast Micromethod® is applicable for the measurement of DNA integrity in not only human biological material for medical purpose, but also in marine invertebrates for genotoxicity assessment (biomonitoring) and the estimation of harmful effects in the ecosystem. This article focuses on a comprehensive description of the method and its uses, and offers detailed descriptions and insights into samples and chemicals preparation, fluorescence analysis and calculation of DNA integrity rate. The results and features of many provided studies using this method are discussed, in order to elucidate advantages and imperfections of this analytical technique and to highlight approaches that may allow its further development.

Contents

Preface		**vii**
Chapter I	Progress in Somatic Hypermutation Research *Hong Ming Shen*	**1**
Chapter II	Factors Leading to the Induction and Conversion of DNA Damage into Structural Chromosomal Aberrations *M. V. Di Tomaso, W. Martínez-López , L. Méndez-Acuña, L. Lafon-Hughes and G. A. Folle*	**55**
Chapter III	Pesticides as Genetic Damage Inducers *Carla Costa, João Paulo Teixeira and Olga Mayan*	**111**
Chapter IV	DNA Damage Related to Exposure to Oil Spills: The Prestige Experience *Beatriz Pérez-Cadahía, Josefina Méndez, Eduardo Pásaro, Vanessa Valdiglesias and Blanca Laffon*	**165**
Chapter V	Differentiated Cells Play Favorites: Dissecting the Mechanisms of Discrimination in DNA Repair *Thierry P. Nouspikel*	**199**
Chapter VI	Functional Roles for Pro-Apoptotic Kinases on Nuclear Targeting and Induction of Apoptosis in Response to DNA Damage *Kiyotsugu Yoshida*	**227**
Chapter VII	How does Tumor Suppressor FHIT Modulate Oxidative Stress and DNA Damage Checkpoints in Early Cancer? *Hideshi Ishii and Toshiyuki Saito*	**243**

Chapter VIII Biological Significance of DNA Damage Checkpoint and the Mode of Checkpoint Signal Amplification **253**
Motohiro Yamauchi, Keiji Suzuki, Yasuyoshi Oka and Shunichi Yamashita

Chapter IX Cobalt Treatment Suppressing Mitochondrial DNA Damage Caused by Acute Exposure of H_2O_2 **261**
Yasutomo Nomura, Zhonggang Feng and Takao Nakamura

Chapter X Molecular Basis of Guanine Oxidation under UV-A/VIS Radiation and its Biological Effects **271**
Katsuhito Kino, Hiroshi Sugiyama and Hiroshi Miyazawa

Chapter XI New Advances on DNA Fragmentation **277**
Thamara Viloria, Nicolás Garrido, Jose Luis Fernández, José Remohí, Antonio Pellicer and Marcos Meseguer

Chapter XII Sperm Chromatin Damage **297**
Monica Muratori, Sara Marchiani, Valentina Tocci, Gianni Forti and Elisabetta Baldi

Chapter XIII Biological Meaning and Clinical Relevance of Sperm DNA Fragmentation in Assisted Reproduction **317**
Nicoletta Tarozzi, Marco Nadalini and Andrea Borini

Chapter XIV Comments on the Clinical Value of Determination of Sperm DNA Fragmentation **341**
José Luis Fernández, Dioleyda Cajigal and Jaime Gosálvez

Chapter XV Assessing the Fast Micromethod® as a Tool in DNA Integrity Analysis **349**
Željko Jakšić

Chapter XVI Unconventional Use of the Comet Assay to Identify and Classify Different Nuclear Phenotypes in Cell Cultures **371**
Alessandra Mocali, Lisa Giovannelli, Vanessa Pitozzi, Michela Jacomelli, Cristina Cellai and Francesco Paoletti

Chapter XVII Bromodeoxyuridine-Labeling and DNA Repair, when Studying Neurogenesis **377**
Philippe Taupin

Index **381**

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Chapter I

Progress in Somatic Hypermutation Research

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Abstract

Somatic hypermutation (SHM) in the immunoglobulin (Ig) variable (V) region and a few other genes in B lymphocytes is a unique process that mainly occurs in germinal centers (GCs). The process is essential for the immune defense, but it has been implied to be tumorigenesis. Here I review progress made in the past decades in unraveling the molecular basis of SHM. SHM was first visualized in the Ig protein and then detected at the DNA level. It starts 100 – 200 bp downstream of the promoter, reaches a peak in the V region and is gradually diminished after the peak. Transcription is generally thought to be required for the SHM process. Although the Ig enhancers are crucial for SHM in the transgenic mouse model, the Ig promoter or the V region sequence can be replaced with a non-Ig one. The preferences of C,G transitions versus transversions, A versus T mutations, and mutations at hotspots indicate that the mutation process is not random, suggesting that there is a mutator factor responsible for the mutations. The mutator factor is activation-induced cytidine deaminase (AID). The enzyme is a single-stranded DNA deaminase and is able to directly attack DNA using tagged AID proteins purified either from insect cells or from bacteria. It preferentially targets WRC hotspots. Moreover, AID acts on supercoiled DNA and has deamination activity on both DNA strands. In vivo, the AID protein is phosphorylated and associated with RPA in order to efficiently deaminate cytidine during transcription. Several major DNA repair pathways have been screened for a possible role in the mutation process. Among these, nuclear excision repair (NER) is apparently unrelated to SHM, while mismatch repair (MMR) and base excision repair (BER) are involved in the mutation process by expanding the C/G>T/A mutation spectrum caused by AID to C,G,A,T transitions/transversions with the help of error-prone DNA polymerases (pols). Typically, pol η generates A,T mutations during MMR. Although much has been learned regarding the AID function, how AID targets DNA

during transcription and why it only attacks Ig and a few other genes remain relatively unknown. Two SHM models are discussed in this review.

Introduction

The somatic hypermutation (SHM) process in immunoglobulin (Ig) genes in B cells dramatically diversifies the antibody repertoire and plays an important role in the immune responses. The process and the following positive selection create high affinity antibodies against foreign pathogens to effectively neutralize them. The frequency of SHM is extremely high, with estimates of up to several percent in the IgV region, greatly exceeding the mutation rate in mammals (~10^{-8} - 10^{-9}/base/generation). Various genetic defects can shape the mutation pattern or reduce the mutation efficiency, which is frequently accompanied with abrogation of Ig gene class switch recombination (CSR) activity, leading to severe reductions of host immunity against foreign pathogens. Besides the clearly desired and beneficial outcome of SHM for the host defense, the process may also trigger aberrant SHM, which may lead to tumorigenesis. Thus, understanding what is the mechanism of the SHM process is of clear importance for both basic and clinical research.

It has been several decades since SHM was for the first time observed. There is substantial evidence that SHM which requires transcription is caused by activation-induced cytidine deaminase (AID), and followed by error-prone DNA repair by two major DNA repair machineries: mismatch repair (MMR) and base excision repair (BER). However, the question what is the bait that lures AID to target Ig and a few other genes remains unanswered. In this review, I will summarize studies of the molecular basis underlying SHM in past decades as the studies significantly advance our knowledge about DNA damage and repair and will also discuss some SHM models that may shed light on our future research on the SHM mechanism.

In this review, I have tried to include as many references as possible. Nevertheless, there are so many publications that can be dug out using the key word hypermutation, which are obviously not included in the abstracts without the term hypermutation. If I have missed a golden apple(s) in the SHM field, I would like to apologize for it. Since I am concentrating on SHM, I refer to CSR only where it is related to SHM.

Observation of SHM

The term hypermutability of Ig genes first appeared in Lederberg's publication in 1959 in which the mutations in the Ig genes are from random assembly of DNA during cell proliferation (Lederberg, 1959). Later, Brenner and Milstein proposed a hypothesis showing that a cleaving enzyme (endonuclease) cuts one of the DNA strands, an exonuclease trims the DNA to create a gap, and a DNA polymerase repairs the damaged DNA and leaves an error(s) on the newly synthesized region. DNA replication then fixes the error(s) (Brenner and Milstein, 1966). However, the question whether SHM exists in the Ig genes was not answered until a subsequent study. In 1970, Weigert and his colleagues showed strong

evidence that the λ light chain gene product was mutated in several human tumor cell lines by analyzing the Ig protein sequences (Weigert et al., 1970). The mutations were accumulated in three regions now called complementary determinant regions (CDR), suggesting that mutations were positively selected (Weigert et al., 1970). However, as Gearhart pointed out in her recent review (Gearhart, 2006), in the early 1970's, there were opposite hypotheses: the somatic model by Weigert et al as aforementioned and the germline model which believed that what appeared to be mutations was polymorphisms in the Ig genes. If the germline model was correct, each B cell should express many Ig genes. However, a hybridization experiment using mouse Igκ mRNA to hybridize mouse liver DNA showed that there were not enough Ig genes to support the germline model (Tonegawa et al., 1974). The landmark work done by Hozumi and Tonegawa revealed that one constant (C) region is recombined with one variable (V) region in a lymphoma cell line (Hozumi and Tonegawa, 1976) so that one V region is expressed in a single B cells. Thus, the germline model was proved not to be correct. After several new technologies, such as Southern blot (Southern, 1975) and DNA sequencing (Maxam and Gilbert, 1977; Sanger and Coulson, 1975), were available in the 70's, several labs directly determined SHM in the Ig genes. The results provided overwhelming evidence that SHM occurs in both the Ig light chain (IgL) and the Ig heavy chain (IgH) at the genomic DNA level (Bernard et al., 1978; Crews et al., 1981; Gearhart et al., 1981; Selsing and Storb, 1981; Sims et al., 1982; Teillaud et al., 1983). However, the precise course leading to the mutations in the Ig genes was not elucidated.

Cells in SHM studies

Myeloma cells

In early SHM studies, myeloma cell lines were widely used for mutation analysis. Myeloma cells are tumor cells derived from plasma cells. Since each myeloma tumor line is derived from the same progeny and secretes antibodies, it was very useful for the SHM studies, especially when DNA sequencing was not available. In addition, mutations observed in myeloma cells were accumulated in the IgV region but not in the IgC region, which is in line with the SHM distribution. By analyzing mutations in IgH and IgL chains secreted from mouse myeloma cells, Coffino and Scharff were able to calculate the mutation rate that reached 1.1×10^{-3} per cell per generation (Coffino and Scharff, 1971), which fits the current estimates that the mutation frequency is about $10^{-4} - 10^{-3}$/base pair/generation (Allen et al., 1987; Milstein, 1991; Weigert, 1986). In the early 80's, Selsing and Storb showed that a restriction enzyme site between a number of other mutations was present in the rearranged IgVκ167 region in a myeloma cell line, but not in the germline IgVκ167 region, confirming that SHM exists (Selsing and Storb, 1981).

Hybridoma cells

In 1975, Kohler and Milstein (Kohler and Milstein, 1975) established hybridoma cells by fusing myeloma cells with mouse spleen cells after mice were immunized with sheep red blood cells (SRBC). Each hybridoma secretes one form of antibody so called monoclonal antibody with specific sensitivity and activity against a single antigen. From a single preparation, one is able to isolate many hybridoma cell lines which can bear various mutations in the Ig variable (V) regions from different families. The establishment of hybridomas dramatically impacted on modern basic biological and clinical research. This technique has also been used in SHM studies. The advantages of hybridoma cells comparing to myeloma cells is that the Ig protein and DNA sequence of interest are from spleen cells and mutations directly reflect the true SHM process. There are potentially unlimited resources using hybridoma cells to search for the SHM mechanism. One of the most notable features is probably that one can compare mutations in the same mouse from the same immunization. Thus, IgG molecules were found to contain more mutations than IgM in mice (Gearhart et al., 1981). The hybridoma technique was also applied in studies of the roles of SHM in Ig diversity (Sims et al., 1982; Teillaud et al., 1983), antibody affinity maturation (Griffiths et al., 1984), and clonal expansion of antibodies (Sablitzky et al., 1985).

Nevertheless, creation of hybridoma cell lines is time-consuming. In addition, generation of hybridoma does not discriminate mutating B cells from naïve B cells so that hybridoma cell lines generated from immunized mice are composed of both mutating and naïve B cells. The naïve B cells usually carry low or no mutation in IgV regions. Thus, if one desires to acquire a high mutation frequency in Ig genes, this experimental model may not be the first choice now. One way to speed up SHM studies is to directly find hypermutating cells in mouse after immunization of mice.

Germinal center B cells

In 1980, Rose et al. found that mouse germinal center (GC) B cells strongly bind peanut agglutinin (PNA) but other B cells bind PNA with low affinity (Rose et al., 1980). Later, GC B cell function had been identified by Liu et al. and Jacob et al. independently, showing that GC B cells respond to T-cell dependent antigens (Jacob et al., 1991; Liu et al., 1991). GC development and maintenance rely on both T cells and follicular dendritic cells (FDCs) (Liu et al., 1996; McHeyzer-Williams, 2003). In brief, in response to foreign antigens, B cells sample the antigens and move to the T cell zone. After digesting the antigens, the B cells present short peptides on their cell membrane, thereby becoming antigen presenting cells. Antigen-primed T cells that recognize the antigen in the groove of class II MHC molecules on the membrane of B cells conjugate the B cells at the boundary between B cell and T cell zones. The activated B cells move either to extrafollicular areas or to B cell follicles, and in the B cell follicles, B cells proliferate and form GCs (Allen et al., 2007a). Normal mice without immunization have few GCs in their spleen. Therefore, in order to collect enough GC B cells mice need to be immunized. There are several useful haptens that can be applied for induction of the SHM process in GC B cells, including 2-phenyl-oxazolone (phOx) (Griffiths