CHAPTER 12.

The role of genotoxicity in carcinogenesis

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The process of mutagenesis

The process of agent-induced mutagenesis consists of three parts: the induction of DNA damage, the sensing of the DNA damage by the cell (the DNA damage response), and the processing of the DNA damage by the cell, which may or may not result in a mutation. A key underlying concept is that mutagenesis is a cellular process, frequently involving DNA replication. Another key concept is that there is a distinct difference between DNA damage and mutation. Thus, mutagens, despite what their name suggests, generally do not produce mutations; instead, mutagens produce DNA damage, and they might more appropriately be called DNA-damaging agents. Instead, it is the cell that produces the mutation – either through faulty DNA repair of the mutagen-induced or spontaneous DNA damage, or by replicating past the unrepaired DNA damage, thereby introducing a replication error (Shaughnessy and DeMarini, 2009).

A description of the process of mutagenesis begins with the induction of DNA damage by an endogenous or exogenous event. Examples of DNA damage are DNA adducts (i.e. a molecule bound covalently to DNA) and single- or double-strand breaks (i.e. breakage of the phosphodiester backbone). Other types of DNA damage are oxidized or fragmented bases and the intercalation of a molecule between a pair of bases. Again, DNA damage is itself not a mutation and generally does not alter the linear sequence of nucleotides. A mutation is defined as a change in the sequence or number of nucleotides in the DNA.

When DNA damage occurs, the cell detects it by means of the DNA damage response system and determines how it will be processed; the DNA damage response includes DNA repair and apoptosis pathways, which are described in detail by Ciccia and Elledge (2010). The DNA damage response can mediate the repair of the damage, attempt to repair the damage but instead process it into a mutation, or direct the cell to undergo apoptosis. Another

possibility is that the damage is not repaired at all, and when the cell replicates, the DNA polymerase correctly bypasses the damage, resulting in a normal DNA sequence.

The cell can process DNA damage into three general classes or types of mutation: gene mutation (mutations that occur within a gene), chromosomal mutation (mutations involving more than one gene, typically called chromosomal aberrations), and genomic mutation (mutations involving the whole genome – generally aneuploidy, which is the gain or loss of a whole chromosome). The standard definition of a gene refers to a segment of DNA that codes for an mRNA that codes for a protein. The recent Encyclopedia of DNA Elements (ENCODE) project indicates that at least 80% of the human genome is transcriptionally active, but only a small proportion of the expressed regions code for protein (Maurano et al., 2012).

In the context of the process of mutagenesis described above, the term "mutagen" refers to an agent that can induce DNA damage that the cell processes into a mutation. The more general term "genotoxin" refers to an agent that induces DNA damage that may or may not be processed by the cell into a mutation. Some assays for genotoxicity, for example, measure only DNA damage, such as ^{32}P-postlabelling and the comet assay, whereas other assays measure mutation, such as the *Salmonella typhimurium* reverse mutation test, the *Hprt* gene mutation assay in Chinese hamster ovary cells, and transgenic mouse mutation assays. Thus, finding that an agent induces DNA damage would permit it to be called genotoxic, and showing that the agent also induces mutations in a mutation assay permits it to be classified as mutagenic.

A brief history of the nexus between mutagens and carcinogens

As reviewed by Claxton et al. (2010), there was little direct evidence for the role of mutagenesis in carcinogenesis until the early 1970s, and before that time only a few carcinogens had been shown to be mutagens (Burdette, 1955). Indeed, it is surprising to recall that at the time it was somewhat bold to propose that there was any direct connection between the two processes (Miller and Miller, 1971; Knudson, 1973). Many studies in the 1950s and 1960s showed binding of carcinogens to nucleic acids (Wiest and Heidelberger, 1953; Brookes and Lawley, 1964). However, before 1972 there was no direct proof that the electrophilicity of some chemical carcinogens had a necessary role in the potential mutagenic activity of such compounds, or even that DNA, as opposed to protein, was the ultimate target of carcinogens (Miller, 1970).

Although sound theoretical reasons had been proposed to support the notion that carcinogens might act through a mutagenic mechanism, a clear demonstration of this connection did not yet exist (Miller and Miller, 1971). Thus, binding to DNA of metabolites of carcinogens had been identified, but there were no data to show that these DNA adducts were processed into mutations or that mutations themselves played a role in carcinogenesis. Consequently, mutagenesis was viewed at that time as an equally plausible mechanism for carcinogenesis, along with epigenetic changes (Miller, 1970; Miller and Miller, 1971), altered expression of an integrated viral genome (Todaro and Huebner, 1972), and alteration of immunological factors by carcinogens, permitting the formation and growth of tumours (Baldwin, 1973). As time has shown, all of the above-mentioned mechanisms can play a role in the carcinogenic process, especially in the light of the accumulating evidence for the important role of epigenetic mechanisms (Baylin and Jones, 2011).

How did the paradigm shift occur that showed a connection between mutagenesis and carcinogenesis? The first screening studies to test the hypothesis that some carcinogens might also be mutagens were performed by Demerec et al. (1951) in *Escherichia coli* and then by Szybalski (1958), who tested more than 400 compounds in *E. coli*. Although additional testing proceeded throughout the 1960s in a variety of systems in bacteria, fungi, and mammalian cells, few carcinogens other than the direct-acting alkylating agents were found to be mutagens, leading to the conclusion that carcinogens were generally not mutagenic.

However, this view began to shift when Malling (1966) combined a chemical hydroxylating mixture with the carcinogens diethylnitrosamine and dimethylnitrosamine, which were not mutagenic in vitro, and showed that the resulting metabolites were mutagenic in the fungus *Neurospora crassa*. Malling (1971) then prepared an enzymatic activation system composed of the supernatant from mouse liver homogenate centrifuged at 30 000*g* (microsomes) plus cofactors, and showed that dimethylnitrosamine was mutagenic in *S. typhimurium* in a liquid suspension assay in the presence of this activation mixture. Additional evidence that carcinogens could be mutagens

after mammalian metabolism was provided by Legator and Malling (1971) with the host-mediated assay.

Ames et al. (1972) introduced the use of the plate incorporation assay in *Salmonella* and demonstrated that DNA-reactive metabolites of known carcinogens were direct-acting mutagens. The connection between mutagenesis and carcinogenesis was extended when Ames et al. (1973) combined a rat liver homogenate centrifuged at 9000*g* (S9 fraction) plus cofactors prepared as described by Garner et al. (1972) with *Salmonella* and a variety of rodent carcinogens then considered to be non-mutagenic in the plate incorporation assay and showed that these carcinogens were, in fact, mutagenic. Additional refinements of the *Salmonella* tester strains and the conduct of multiple testing studies, involving not only *Salmonella* but also other test systems (Tennant et al., 1987), resulted in the current recognition that many carcinogens, by themselves or after metabolic activation, are mutagens, and that mutagenesis is a critical feature of carcinogenesis.

Despite the recognized importance of mutagenicity as a part of cancer induction and progression, by the 1990s it appeared that many rodent and human carcinogens were, in fact, not clearly mutagenic or genotoxic. Some operate through receptor binding, which can result in an alteration in gene expression, often leading to increased cell replication. However, an analysis of a set of so-called non-genotoxic carcinogens found that most of them were, in fact, genotoxic (inducing DNA damage and/or mutation) when tested adequately for both gene, chromosomal, or genomic (aneuploidy) damage and mutation (Jackson et al., 1993). Indeed, a comprehensive analysis showed that more than 90% of the IARC Group 1 chemical carcinogens are genotoxic (Waters et al., 1999).

The current genetic toxicity test battery is based on this relationship between mutagenesis and carcinogenesis. Consequently, mutagenicity assays continue to be used as a potential screen for carcinogens, and the results are used for regulatory purposes throughout the world (Eastmond et al., 2009). For example, a positive result in the *Salmonella* mutagenicity assay indicates a 70% probability that the test chemical is a rodent carcinogen (Zeiger, 1998). When a randomly selected set of 100 organic compounds was tested in the *Salmonella* mutagenicity assay, about 20% of them were positive (Zeiger and Margolin, 2000). Thus, out of an estimated 80 000 such compounds in commercial use, 16 000 (20%) may be positive for mutagenicity in the *Salmonella* mutagenicity assay, and 11 200 (70%) of those may be potential rodent carcinogens.

Mutations in tumours

Soon after the discovery of the correct number of human chromosomes (46) by Tjio and Levan in 1956 (Gartler, 2006; Harper, 2006), cytogenetic studies began to show that tumours (specifically leukaemic cells) had higher frequencies of chromosomal aberrations than did normal cells (Nowell and Hungerford, 1960). A decade later, the development of quinacrine fluorescence and Giemsa staining enabled the first discovery that a specific chromosomal aberration was associated with a specific type of leukaemia (Rowley, 1973). As evidence accumulated that chromosomal aberrations were present at high frequencies in tumours, DNA sequencing methods were introduced in 1977 (Pettersson et al., 2009), which provided the technical means to directly determine the presence and types of mutations in any gene or chromosome.

DNA sequencing of mutations induced in selected genes by a limited number of mutagenic carcinogens in microbes in the 1980s and in mammalian cells and tumours in the 1990s began to show that any particular mutagen produced an array of mutations and that these varied among the genes and cells examined. A variety of mutagens produce similar mutation spectra, and the predominant base substitution that an agent induces in one system is generally the same one that the agent produces predominantly in all other systems across the phylogenetic scale, from bacteria to humans (DeMarini, 1998, 2000). Thus, in terms of the predominant base substitution produced by agents, there is concordance across species in that the DNA damage induced by a particular agent is processed similarly by a wide range of species.

With regard to mutations in tumours, generally elucidated without reference to any exogenous causative agent, the technology in use from the 1980s until the early 2000s permitted the determination of mutations in only a few cancer-related genes, such as *TP53* and *KRAS*. The first gene mutation in a human tumour was determined in 1982 (Reddy et al., 1982), and by the end of the 20th century, there was clear evidence that some tumours had mutations in certain oncogenes and tumour suppressor genes that could be associated with the types of mutations produced by the carcinogen associated with the induction of the

tumour – both in rodents and in humans (Dogliotti et al., 1998; Hainaut and Wiman, 2009). Examples include CC → TT mutations in the *TP53* gene in skin tumours associated with exposure to sunlight, G → T mutations in codon 259 of the *TP53* gene in liver tumours associated with exposure to aflatoxin B_1 (Ceccaroli et al., 2015), A → T mutations in the *TP53* gene in tumours of the upper urinary tract associated with exposure to aristolochic acid (Grollman, 2013), and the different locations of G → T mutations in the *TP53* and *KRAS* genes in lung tumours associated with exposure to cigarette smoke (IARC, 2012) or to emissions from smoky coal (DeMarini et al., 2001). There are about 20 carcinogens that are known to produce either unique or distinctive mutation spectra in tumours linked epidemiologically to specific exposures in humans (Ceccaroli et al., 2015).

With the advent of next-generation DNA sequencing, exome and genome sequencing of tumours was first reported by Wood et al. (2007) and Parsons et al. (2008). These initial studies revealed that tumours had large numbers of mutated genes; however, it appeared that only a few (six to eight) genes were involved directly in the carcinogenic process. As few as three driver gene mutations are required for the development of lung cancer or colorectal cancer (Tomasetti et al., 2015). Large-scale sequence analysis of the genomes of thousands of human tumours has identified new genes that are important for cancer and new mutational signatures that are specific to particular tumour types and subtypes (Hoang et al., 2013; Alexandrov and Stratton, 2014).

As discussed elsewhere in this Scientific Publication (see Chapter 11, by Stewart, and Chapter 19, by Caldwell et al.), cancer is a genetic disease that proceeds by a type of Darwinian evolution (Hanahan and Weinberg, 2011). In this way, changes in gene function (by mutation) and in gene expression (by epigenetic mechanisms) that result in a cell having a specific growth advantage may be selected for in certain tissue microenvironments (Hanahan and Weinberg, 2011; Solomon et al., 2011; Whitfield and Soucek, 2012).

Stratton (2011) estimated that most human tumours contain 1000 to 10 000 base substitution mutations; tissues exposed more directly to the environment, such as the lung and the skin, have tumours with more than 100 000 mutations. However, only about 400 genes (~2% of the coding genome) appeared to be involved directly in tumorigenesis; the rest were likely to be passenger mutations, i.e. mutations not related to the carcinogenic process and possibly resulting from the genomic instability of the tumour (Bozic et al., 2010).

Genome or exome sequencing has identified 20 distinct mutational signatures among human tumours and confirmed that tumours of the most "protected" organs (e.g. the brain) have only a few mutations, whereas those in organs exposed more directly to the environment (e.g. the lung and the skin) have thousands of mutations (Alexandrov et al., 2013). The vast majority of mutations in tumours are base substitutions (Vogelstein et al., 2013). Only one or two mutated genes appear to be required for some haematopoietic tumours, whereas at least five or six are required for solid tumours (Stratton, 2011). In addition, all tumours have many genes with altered gene expression (Baylin and Jones, 2011).

A recent discovery is "shattered" chromosomes in tumours, a phenomenon termed chromothripsis, which results in massive chromosomal rearrangements in 1–3% of human tumours (Stephens et al., 2011). Studies indicate that these shattered, highly rearranged chromosomes may appear exclusively in micronuclei (Crasta et al., 2012; Maher and Wilson, 2012), providing a new insight into the potential role of micronuclei in tumours (Hatch and Hetzer, 2015).

As evidence had accumulated that mutation and mutagenesis are essential features of carcinogenesis, the notion that tumours might be monoclonal became popular, because of the monoclonality of haematopoietic malignancies and because this notion appeared to support the prevalent initiation–promotion model of carcinogenesis. However, as molecular analyses of tumours became more sophisticated, it soon became clear that tumours are not monoclonal and that they are, in fact, highly heterogeneous (Parsons, 2008).

The most exquisite evidence for this has been provided by Gerlinger et al. (2012), who showed that approximately two thirds of all somatic mutations were not present in all regions of a set of kidney tumours analysed by a combination of exon sequencing, chromosomal aberration analysis, and mRNA expression analysis. Recently, Martincorena et al. (2015) demonstrated that this heterogeneity is established early on, by showing that physiologically

normal human skin contains a patchwork of thousands of evolving clones, with more than one quarter of such cells having cancer-causing mutations.

Although there is now also overwhelming evidence for the essential role of epigenetic changes in the carcinogenic process (Grønbaek et al., 2007; Baylin and Jones, 2011) and for the fact that many carcinogens can induce such changes (Ceccaroli et al., 2015; Nicolaidou and Koufaris, 2015), as discussed below there is emerging information that mutation itself might underlie some, if not most, of these epigenetic changes.

There are three primary epigenetic mechanisms by which cells regulate gene expression: methylation of DNA (Hsiao et al., 2009), modifications of histones (Ellis et al., 2009), and binding of microRNAs and other non-coding RNAs to the genome or to other RNAs (Garzon et al., 2009). However, studies have shown that mutations in genes involved in these three processes may be the basis for many of the epigenetic events mediated by these mechanisms (You and Jones, 2012). For example, mutations in specific chromatin-modifying genes appear to occur in specific cancers, such as in *JARID1C* in renal cancer, in *SMARCA4/BRG1* in lung cancer, and in *ARID1A* in ovarian cancer (Jones et al., 2010). Also, mutations in the DNA methyltransferase genes *DNMT1* and *DNMT3A* are found in colorectal cancer or acute myeloid leukaemia, the histone lysine methyltransferases or demethylases *HK4*, *H3K9*, and *H3IK27* are mutated in kidney cancer and colon cancer, and the histone acetyltransferases *H3K18* and *H3K27* are mutated in acute lymphoblastic leukaemia (Peltomäki, 2012; Ryan and Bernstein, 2012). Although epigenetic changes per se are not mutations because the sequence of nucleotides has not been changed, as evidenced above, mutation may be the basis for some epigenetic events.

Models of agent-induced carcinogenesis

Data generated in recent years have led to a reconsideration of the dichotomy between so-called non-genotoxic versus genotoxic carcinogens (Waters et al., 1999) and indicate that some epigenetic events may have a mutational basis (You and Jones, 2012). In addition, chronic inflammation, which is associated with increased cancer risk (Colotta et al., 2009), causes DNA damage (etheno-base lesions and other exocyclic DNA adducts) that appears to be the basis for the increased risk, as demonstrated by the fact that repair of the damage by base excision repair enzymes (alkyl glycosylases) reduces the risk of cancer (Calvo et al., 2012). Indeed, an analysis of a dozen human studies found strongly increased risks of cancer among individuals with high levels of DNA adducts relative to those with low levels, and the cancer risks were even higher for the group with high adduct levels when other risk factors, such as infection and inflammation, were taken into account (Poirier, 2012). As noted in Chapter 19, by Caldwell et al., host susceptibility factors modulate all of these events and are a critical element in the overall cancer risk.

Within the context of both the initiation–promotion model of carcinogenesis and the "hallmarks" of cancer (Hanahan and Weinberg, 2011), these data have led to the view that (i) cancer is essentially a genetic disease and (ii) an agent that causes cancer induces alterations in gene function (by mutation) and/or gene expression (by epigenetic changes), either by direct interaction with DNA or chromatin or by indirect mechanisms, such as through generation of reactive oxygen species, inflammation, and/or receptor-mediated interactions. These considerations suggest that carcinogens must be genotoxic in the broadest sense of the term, i.e. they damage DNA or alter its expression either directly or indirectly, leading to a change in function or expression of genes. Such changes in the appropriate genes with promotion through cell replication and selective pressure can then lead to a tumour. For colorectal tumours this concept has been characterized as the "Big Bang" model for tumour growth, in which tumours start early on producing mixed subclones that are not subject to stringent selection, thus explaining the heterogeneity of tumours (Sottoriva et al., 2015).

This greater appreciation for how chemical, physical, and biological agents may induce cancer leads to a model for agent-induced carcinogenesis that integrates portions of the classic initiation–promotion model with elements of the hallmarks of cancer. Such a model would envision a carcinogenic agent establishing the process by either genetic or epigenetic mechanisms that cause changes in gene function and expression, resulting in the plethora of characteristics of cancer cells, i.e. the hallmarks of cancer: mutations in key oncogenes, altered gene expression, changes in cell signalling, altered cell growth, evasion of apoptosis, sustained angiogenesis, increased genomic instability, and eventual metastasis. Much of this can be modulated by various sus-

ceptibility factors, including genetic or epigenetic factors, as well as by a large number of environmental and lifestyle factors (see Chapter 19, by Caldwell et al.). However, a genotoxic carcinogen may not necessarily cause cancer via a genotoxicity mechanism alone or predominantly, and further mechanistic studies are needed to delineate the carcinogenic mechanisms of any particular agent.

This generalized model no longer makes a distinction between initiation and promotion, which was an operational model derived largely from mouse skin-painting studies. Similarly, it does not divide carcinogens into genotoxic and non-genotoxic categories. Instead, an integrated model of agent-induced carcinogenesis as described above emphasizes the ability of the carcinogen (chemical, physical, or biological) to alter gene structure (by mutation) and/or gene expression (by genetic or epigenetic changes), leading to functional changes in the genome that manifest themselves through changes in cell signalling, altered cell growth, and genomic instability, resulting in the hallmarks of cancer, with susceptibility factors modifying various aspects of these processes and outcomes. The importance of epigenetic changes, cell signalling, and tissue–cell interactions have suggested alternative models to the somatic mutation theory of cancer, such as tissue organization field theory (Baker, 2015) and tissue programming theory (Burgio and Migliore, 2015).

Summary

Work since the 1970s demonstrated that many carcinogens are either directly or indirectly genotoxic or mutagenic and/or alter gene expression. Analyses of tumours, first by cytogenetic methods in the 1970s, then by single-gene analysis in the 1990s, and most recently by exome or whole-genome sequencing, have demonstrated clearly that mutagenesis is a central feature of carcinogenesis. Thus, it is not surprising that more than 90% of the known human chemical carcinogens (IARC Group 1) are positive in conventional short-term tests for genotoxicity (Waters et al., 1999).

Cancer is now recognized as an essentially genetic disease, with carcinogens causing genetic damage and/or changes in gene expression either directly or indirectly. This recognition should prompt a reconsideration of the distinction between genotoxic and non-genotoxic carcinogens. A generalized model of agent-induced carcinogenesis would no longer make a distinction between initiation and promotion but would instead emphasize the initial effects of the agent that then lead to a series of changes in cell signalling that result in the hallmarks of cancer, with the entire process being modified by a variety of susceptibility factors.

The ability of carcinogenic agents to induce mutation and/or alter gene expression, with either ability being sufficient to initiate the process of tumour formation (Grønbaek et al., 2007; Halazonetis et al., 2008), is now an established feature of agent-induced carcinogenesis. This deeper understanding of the relationship between genotoxicity and carcinogenicity is the culmination of research that provided the first evidence for such a relationship only about 40 years ago.

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