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DNA Damage by Drugs and Radiation: What is Important and How is it Measured?

Stephen J. Whitaker

DNA is the most important target for drug and radiation induced cell killing. The mode of cell killing by cytotoxic drugs and radiation has been derived by correlating the type and quantity of DNA damage induced with lethality. Cytotoxic drugs can be classified by their main mode of action, while ionising radiation causes a range of lesions with the DNA double-strand break (dsb) being the most significant. Strand-breaks are measured from the reduction in the size of DNA molecules following treatment. Molecule size can be derived from the rate that DNA fragments sediment when centrifuged, elute through filters or migrate under electrophoresis. The effect of strand-breaks on DNA loop supercoiling allow a sensitive assay of DNA damage. Specific assays for base damage and drug adducts include changes in chromatographic mobility or binding by specific antibodies. By comparing the levels of damage in the genome overall with damage in specific gene targets, regions susceptible to damage induction, and varying in repair efficiency, have been revealed.
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INTRODUCTION

THE MODE of action of most cytotoxic agents in common use is via DNA damage. Understanding this mode of action can suggest ways to manipulate and increase cytotoxicity (for example by using oxygen mimetic drugs with ionising radiation) and can suggest novel agents to explore. DNA damage occurs by both direct and indirect means leading to cell death or mutagenesis. In most cases the exact mechanisms are not known but are probably common to both cell events. The purpose of this short review is to focus on what is known about the mechanisms of cell killing by genotoxic agents and how DNA damage is measured.

DNA damage occurs spontaneously

A single cell contains all the information required to manufacture, maintain and reproduce the organism from which it is derived. In diploid, eukaryotic cells this information is stored as double-stranded DNA, a macromolecule containing 6×10^9 nucleotide pairs, some 50 cm long if stretched out, which, by winding and supercoiling (the effect seen when a telephone flex is twisted), undergoes a 100 000-fold reduction in length to be packed into a 5 μm nucleus and yet remains accessible for transcription and replication, see Fig. 1 [1]. It is a changing structure; chemical bonds break, bases are lost, and strand-breaks occur. These arise from environmental genotoxic agents (such as natural radiation) and radical damage from oxidising radicals produced during normal metabolic processes. These events are unavoidable and happen thousands of times per hour in each cell [2].

Spontaneous errors occur during DNA replication when DNA polymerases add incorrect nucleotides (due to transient chemical base changes). These events are relatively frequent (approximately 1 in 10^5 bases) but efficient "proof-reading" leaves point mutations with an estimated frequency of only 1 in 10^9 bases. Larger sequence changes occur in meiotic recombination. These changes lead to genetic variety and evolution which may be of positive benefit to the organism, however most damage is detrimental to the cell and a variety of repair mechanisms exist to correct this (see Ross and Brown pp.281–285). In yeast cells more than 30 DNA repair genes have been identified and it is likely many more are given over to DNA repair in mammalian cells. It has been estimated that a human germ-line cell suffers only 20 stable base changes per year from spontaneous errors and damage.

How might DNA damage kill cells?

Cell death is defined as a loss of reproductive capacity which in the laboratory is tested for by clonogenic assay. Damage which interferes with replication would kill cycling cells (proliferative death) while a failure of transcription effects both cycling and

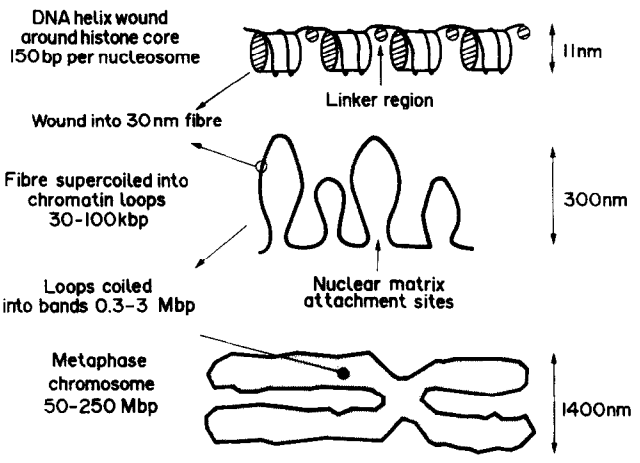

Fig. 1. Some features of DNA packing into a eukaryotic cell nucleus. The nucleosome comprises an octomer of four histone proteins, H2A and 2B, H3 and H4 while H1 (in the linker region) acts to bind the nucleosomes together and twists into a 30 nm fibre. This linker region allows local modification of the degree of winding to facilitate replication and transcription. Cell specific active genes occur in loop domains while housekeeping genes are situated close to the nuclear matrix attachment regions. Replication and transcription occur in this region too.

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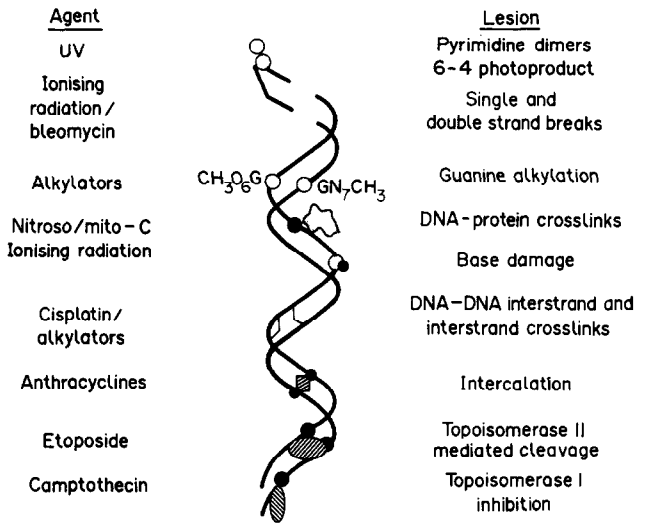

Fig. 2. Major types of DNA lesion caused by cytotoxic drugs and radiation. Some agents cause several types of damage such as ionising radiation and anthracyclines (see text), while others are more specific, such as bleomycin.

deletions lead to a reduction in or altered function of proteins which could reduce cell metabolism and lead to interphase death.

What are the lesions produced in DNA?

A spectrum of lesions similar to spontaneous events is induced in DNA by drugs [3, 4] and radiation [5]. Particular types of lesions are recognised in each class of agent (Fig. 2). Alkylators cause covalent binding of alkyl groups preferentially to the O^6 and N^7 positions of guanine bases (to form drug 'adducts'). If monofunctional (i.e. with one reactive group), base damage and single strand breaks (ssb) can result while if bifunctional (as most commonly used alkylators are), interstrand or intrastrand DNA–DNA crosslinks and DNA–protein crosslinks (dpc) are formed. Cisplatin has a similar crosslinking action to the bifunctional alkylators. The action of other DNA interactive drugs such as doxorubicin and etoposide is less well understood but involves strand breaks mediated by topoisomerase II (an enzyme normally responsible for inducing a controlled double-strand break, dsb, in DNA which allows unravelling of newly replicated strands and controls the degree of loop supercoiling). Strand breaks by bleomycin involve a drug-ferrous iron complex binding to DNA.

Ionising radiations such as charged particles can disrupt atomic structure directly whereas γ-rays and X-rays are indirectly ionising by releasing energetic electrons when these rays are absorbed. This absorbed energy can occur on DNA itself or through oxidising radicals such as $OH^{\cdot}$, produced by radiolysis of nuclear water. Atomic ionisation is rapid, occurring within microseconds of a photon passing through a nucleus. The resulting disruption of chemical bonds leads to a variety of lesions in DNA and associated proteins. For each Gy of energy absorbed by the cell approximately 20000 bases and 1000 sugar groups are damaged, 1000 ssb, 150 dpc, 40 dsb and 30 DNA–DNA crosslinks are produced.

Which lesions are important for cell lethality?

The identification of significant lesions can be achieved by correlating cell killing with the production of different lesions within one cell type or by comparing the levels of damage produced in different cells of varying sensitivity to the agent. For example several radiosensitive mutants of Chinese hamster cells have been derived [6] which are deficient in dsb rejoining indicating the significance of this lesion.

Significant lesions could firstly include the type of lesion. There is experimental evidence that the dsb correlates most closely with ionising radiation induced cell killing [7, 8]; cisplatin produces intra- and inter-strand DNA–DNA crosslinks but it is the former which seem to be more lethal. It is not known whether these lesions are less repairable or inherently more lethal due to concomitant damage to other parts of DNA structure. Ionising radiation can deposit energy in clusters causing local multiply damaged sites in DNA [9, 10] which consist of dsb with ssb, base damage and crosslinks. Alternatively it may be a sub-type of lesion which is most lethal; dsb vary in the exact chemical nature of the sugar-phosphate damage which gives rise to the break [11]. Secondly, the initial level of overall damage induced. Studies of human tumour cells have found more radiation induced dsb in radiosensitive cells than resistant cells [12]. Third, the site that damage occurs within the genome. Actively transcribing genes (those 'switched on' in a cell to produce proteins) have been found to suffer more ssb, dpc and base damage from ionising radiation [13, 14], and strand-breakage by bleomycin [15], while transcribing oncogenes are more sensitive to alkylation [16, 17] and more susceptible to etoposide induced topoisomerase II cleavage [18, 19]. Finally the timing of DNA damage relative to the cell cycle. It is known that radiosensitivity of proliferating cells is greatest in mitosis and lowest in late S phase. One explanation is that there are crucial points in the cell cycle where the persistence of DNA damage is irrevocably fatal for the cell [20]. These may reflect the cell cycle control points identified in yeasts at G_1S and G_2M.

Damage correlates with cell death but may not imply causality

The lesion being measured may be produced in similar proportion to the significant lesion. Following UV irradiation the most abundant lesion produced is the cyclobutane dipyrimidine (TT) dimer which correlates with cell killing, but controversy now exists whether a different lesion, the 6–4 photoproduct (which has recently become measurable) is more significant for mutagenesis and cytotoxicity [21, 22].

Similarly, evidence of drug binding to DNA may not prove its mode of action. For example, doxorubicin intercalates into DNA and it was thought cell death followed interruption of replication or transcription. However, DNA strand breaks are also observed with doxorubicin either as an oxidising radical formed by redox reactions in the cell [23], or through topoisomerase II interactions [24]. A further mechanism of doxorubicin cytotoxicity may be via cell membrane interactions with an enzyme involved in conducting messages to the nucleus (protein kinase C) where the drug may not enter the cell at all [25, 26].

Principles of measuring DNA damage

DNA strand breakage can be visualised directly by cytogenetic means as chromosome fragments, gaps and deletions, but these assays often require cell growth and are difficult to apply to a range of cell types. In addition they do not study DNA at the molecular level and may not allow direct conclusions on mechanisms of damage and repair.

There are many molecular assays of DNA damage [27, 28]. Those that measure damage to specific components of DNA (such as bases or phosphate groups) require isolation of DNA from the cell membranes, cytoplasm and organelles using deter-

gents, heat and proteolytic enzymes such as proteinase-K treatment which digests most proteins including the nuclear matrix.

Assays for strand-breaks can be performed on such 'naked' DNA or on DNA which maintains a degree of its native structure. When cells are lysed in a non-ionic detergent and high salt (2M NaCl), DNA is isolated free of loosely attached proteins, such as histones, in the form of loops attached at their bases to the nuclear matrix (Fig. 1), a filamentous structure which acts as a nuclear skeleton. These points of fixation allow the loops to maintain their supercoiling. The resulting complexes are called "nucleoids".

Quantification of these assays requires "labelling" the DNA either with a radioactive nucleotide, (most often accomplished *in vitro* by growing cells in ^{14}C-thymidine supplemented medium), or by measuring the intensity of fluorescence from an intercalating dye such as ethidium bromide. The latter method is more suitable for measuring damage produced *in vivo* or in nonproliferating cells.

Strand-breakage assays

Ionising radiation and bleomycin cause strand-breaks directly. Lesser types of damage can be measured by converting them into strand-breaks; for example base alterations by radiation or drug can be converted into strand-breaks by treating isolated DNA with a bacterial endonuclease which recognises and cleaves DNA at the site of damaged bases.

When DNA isolation is performed at neutral pH the two strands of the helix remain hydrogen-bonded but at alkaline pH (>11), the two strands separate. The pH for lysis thus determines whether double- or single-strand breaks, respectively are measured.

The level of strand-breaks is indicated by the reduction in average size of DNA molecules in test cells compared with untreated controls. Fragment size is measured by its rate of sedimentation (sucrose gradient sedimentation), filtration through a 0.2 micron filter (filter elution) or by electrophoretic mobility (pulsed-field gel electrophoresis, PFGE).

These assays can also be applied to measure the reduced mobility of DNA fragments when DNA–DNA or DNA–protein crosslinks are formed.

It is desirable to study damage induced in the dose range relevant to cell killing. The sensitivity of these assays (defined as the ability to detect damage from small doses over a large dose range), is in part determined by how the DNA is manipulated after cell lysis. Procedures which involve pipetting isolated DNA cause extra fragmentation due to shearing forces. Consequently supralethal doses of cytotoxic drug or radiation have to be used to detect a difference from controls. By lysing cells which are suspended in a matrix of agarose, DNA continuity is maintained and sensitivity increased, as in PFGE.

Assays of DNA with higher order structure maintained

Several assays exist which measure the influence of strand-breaks on the structure of chromatin in the form of 'nucleoids'. When untreated cells are exposed to a dye such as ethidium bromide the chemical intercalates between nucleotides, relaxes the supercoiling of chromatin loops and the nucleoid size increases [29]. With further increase in dye concentration supercoiling is induced in the opposite direction and the loops decrease again in size. Nucleoid size can be measured by sedimentation [30], increased light scattering in a flow cytometer or it can be visualised directly under a fluorescent microscope as a "halo" [31] around the cell. When ssb/dsb are introduced by drugs or radiation there is a dose dependent increase in the maximum halo size and a reduction in the degree of rewinding.

Drug damage

Drug adducts and base damage can be measured by High Performance Liquid Chromatography (HPLC) [32]. A more sensitive method uses antibodies against DNA modified by drug adducts or cross-links and standard peroxidase or fluorescent imunocytochemistry methods to detect binding to test cells [33, 34]. Antibodies exist to cisplatin adducts, *N*-mustard, procarbazine and melphalan and have been used to study *in vivo* drug levels after therapy or accidental exposure, heterogeneity of adduct formation and removal in different tissues, and site specific carcinogenesis.

Heterogeneity of damage

To determine sensitive sites for DNA damage and repair within the genome, the target of interest has to be selectively labelled and compared to the rest of DNA. For example, damage in newly replicated DNA can be identified by growing cells for 2–3 days in ^{14}C-thymidine to uniformly label whole DNA, then exposing cells for 1–2 h ("pulse labelling") with ^{3}H-thymidine, which is then selectively incorporated into nascent DNA. Damage assays can then distinguish between total and new DNA from the different labels. Using this approach, newly replicated DNA has been found to suffer more base [35] and dpc [36] damage than total DNA. Increased levels of ssb and base damage have been found in actively transcribed regions of the genome, determined by comparing overall genomic damage with that part hybridising to a cDNA probe (complementary DNA derived from reverse transcription of messenger RNA which probes for actively transcribing genes, [13]).

It would be most exciting to study the distribution of significant, i.e. lethal lesions in the genome but for radiation-induced dsb this has been hampered by the low sensitivity of previously used assays. With PFGE, to separate DNA fragments followed by Southern blotting (transfer) of DNA, genetic probes can be applied to specific regions. One problem is that a single gene is a small target and supralethal doses of radiation have to be given to detect an effect. Using probes for sequences widely dispersed and repeated through the genome or a probe for an amplified gene (such as methotrexate resistance gene) can overcome this [37].

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Antibody Therapy of Malignancy

M.J.S. Dyer

INTRODUCTION

ANTIBODIES HAVE the specificity necessary to deliver targeted therapy. However, despite their widespread clinical application in other situations, and despite intensive research (29% of the US NCI budget is devoted to biological therapy), antibodies have yet to make a significant impact on the treatment of cancer.

The purpose of this article is to outline the nature of the problems associated with antibody therapy of malignancy and to show how they may be overcome by the use of molecular genetic and protein engineering techniques.

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THE ANATOMY, DISSECTION AND REBUILDING OF ANTIBODY MOLECULES

Antibodies have two functions: first to bind to antigen and then to transmit the appropriate stimuli to the rest of the immune system including T cells, macrophages and complement so that the concerted effects of all the 'natural effector mechanisms' can be brought to bear.

These different functions are performed by discrete areas of the antibody molecule. The molecule is Y shaped, the tips of the Y binding to antigen and the stem eliciting the various effector functions (Fig. 1). Furthermore, each of these functions is encoded in distinct protein 'domains'. This greatly facilitates antibody engineering allowing the transfer of antigen binding domains onto the various effector domains to produce human antibodies and the production of isolated antigen binding