

REVIEW



Track to the future: historical perspective on the importance of radiation track structure and DNA as a radiobiological target

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ABSTRACT

Purpose: Understanding the mechanisms behind induced biological response following exposure to ionizing radiation is not only important in assessing the risk associated with human exposure, but potentially can help identify ways of improving the efficacy of radiotherapy. Over the decades, there has been much discussion on what is the key biological target for radiation action and its associated size. It was already known in the 1930s that microscopic features of radiation significantly influenced biological outcomes. This resulted in the development of classic target theory, leading to field of microdosimetry and subsequently nanodosimetry, studying the inhomogeneity and stochastics of interactions, along with the identification of DNA as a key target.

Conclusions: Ultimately, the biological response has been found to be dependent on the radiation track structure (spatial and temporal distribution of ionization and excitation events). Clustering of energy deposition on the nanometer scale has been shown to play a critical role in determining biological response, producing not just simple isolated DNA lesions but also complex clustered lesions that are more difficult to repair. The frequency and complexity of these clustered damage sites are typically found to increase with increasing LET. However in order to fully understand the consequences, it is important to look at the relative distribution of these lesions over larger dimensions along the radiation track, up to the micrometer scale. Correlation of energy deposition events and resulting sites of DNA damage can ultimately result in complex gene mutations and complex chromosome rearrangements following repair, with the frequency and spectrum of the resulting rearrangements critically dependent on the spatial and temporal distribution of these sites and therefore the radiation track. Due to limitations in the techniques used to identify these rearrangements it is likely that the full complexity of the genetic rearrangements that occur has yet to be revealed. This paper discusses these issues from a historical perspective, with many of these historical studies still having relevance today. These can not only cast light on current studies but guide future studies, especially with the increasing range of biological techniques available. So, let us build on past knowledge to effectively explore the future.

ARTICLE HISTORY

Received 7 June 2017
Revised 22 September 2017
Accepted 27 September 2017

KEYWORDS

Track structure;
microdosimetry; clustered
damage; history; LET

Introduction

Understanding the mechanisms behind induced biological response following exposure to ionizing radiation is not only important in assessing the risk associated with human exposure but potentially identifying ways of improving the efficacy of radiotherapy. In vitro and in vivo exposure to ionizing radiation can induce a wide variety of biological changes, however, the biological effect observed is not only dependent on absorbed dose, but also on radiation quality. It has long been known that differences in the nature of energy deposition between different types of ionizing radiation can affect the resulting initial chemical, biochemical and biological damages. This can ultimately lead to differences in the response of cells, tissues and organisms, such as spectra of mutation and chromosome aberrations following processing of this damage, through to differences in the probability and latency of carcinogenesis, as well as hereditary effects. From a historical perspective, this paper discusses the

importance of spatial and temporal correlation of energy deposition along radiation tracks, on the nanometer (DNA) scale, the micrometer (nuclear) scale and through to the mm (tissue) scale in determining the biological response (Figure 1). The resulting clustering of damage makes it unique compared to other agents and endogenous processes. These radiation tracks vary significantly with radiation quality and understanding the differences in biological effectiveness is becoming increasingly important with the advent of proton and carbon ion therapy and the need to optimize treatment.

The nanometer/DNA scale

A critically important feature of ionizing radiation is that the insult is always in the form of highly structured tracks of ionization and excitation events. As a result, these are highly inhomogeneous in space and time (all interactions from a single track occur within about 10^{-13} s), with a diversity of

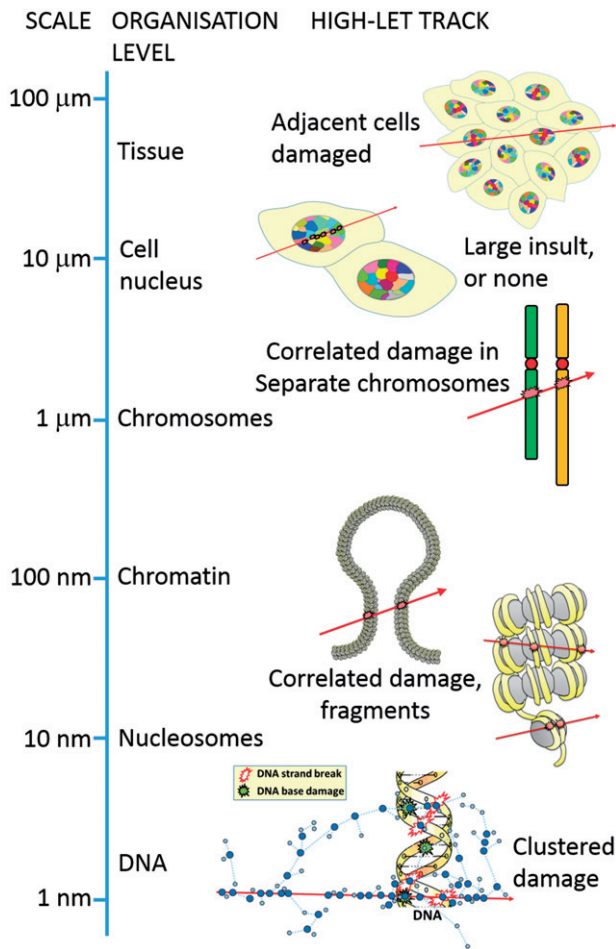


Figure 1. Dimensions and levels of organization over which radiation track structure may be important in determining biological response, with illustrations for high-LET tracks. The track structure for high-LET and low-LET, and corresponding pattern of damage are different across all these levels. Reproduced from "Goodhead DT, Mechanisms for the biological effectiveness of high-LET radiations. *J Radiat Res* 1999, 40:51–13" by permission of the Oxford University Press on behalf of The Japan Radiation Research Society (JRRS), and the Japanese Society for Radiation Oncology (JASTRO).

microscopic features and the spectrum of these events varying with radiation quality. The ability of these differences in microscopic features to influence the biological response was known as early as the 1930s. Early analysis on the link between radiation track structure and the resulting biological response was hampered by the limited description of the radiation tracks. These tracks were often described using the averaged one dimensional term of linear energy transfer (LET) relating to the average energy loss along the track, which was found to be important in determining biological response. For example, early experiments by Barendsen et al. (1963) demonstrated for mammalian cells irradiated with deuterons and alpha-particles that the relative biological effectiveness (RBE) for inactivation increased with LET, increasing to a peak at $\sim 100\text{--}200 \text{ keV } \mu\text{m}^{-1}$ followed by a subsequent decrease at higher LETs (Figure 2).

Even early on the importance of the nanometer scale was recognized. The track structure descriptions were improved by incorporating non-random fluctuations and clustering of ionization events along one-dimensional paths based on cloud-chamber measurements. The concept of clusters of

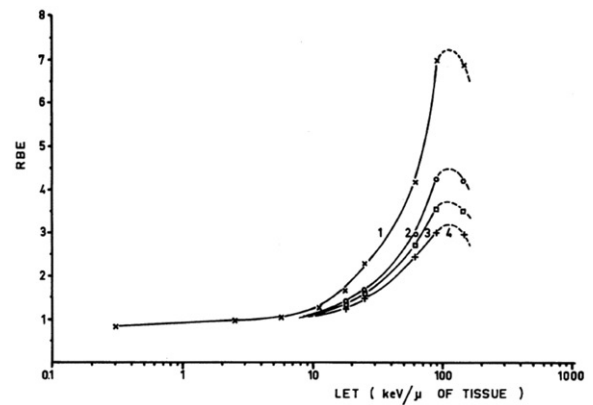


Figure 2. Variation in RBE as a function of LET for clonogenic survival as determined by Barendsen et al. (1963). Curves 1, 2, 3 and 4 corresponds to 80%, 20%, 5% and 0.5% survival respectively (with permission).

ionization being a critical feature of radiation damage was applied to microbial data. Howard-Flanders (1958) and Brustad (1962) were able to model data assuming a lethal lesion required a minimum of 1–10 ionizations within of 3–10 nm. This approach was subsequently applied to mammalian data, Barendsen (1964) found that the low-LET response fitted best assuming 10 or more ionizations in 7 nm, while the high-LET required 15 or more ionizations in 10 nm. This approach was extended by Goodhead et al. (1980), by assuming there were two types of lesions. One lesion requiring 3–9 ionizations in 3 nm (with a low probability of effect) which dominates for low-LET radiation and the other lesion requiring 10 or more ionizations in 3 nm (with a high probability of effect) corresponding to a high-LET lesion. Although the approach made no assumptions about the nature of the target, the analyses pointed to the target dimensions being of the order of a few nanometers.

These simple one-dimension descriptions were subsequently developed to reflect three-dimension information; these included splitting the track into 'spurs', 'blobs' and 'short tracks' (Mozumder and Magee 1966) or using the amorphous track description of charged particles with an average radial dose profile around an average track 'core' (Butts and Katz 1967). In the 1970s, the advance in computer power made it possible to use Monte Carlo techniques to perform an interaction by interaction simulation of the radiation tracks produced by individual particles, giving full 3D information on stochastic energy deposition events. Goodhead and Brenner (1983) were the first to apply track structure simulation to radiobiological data for a range of X-ray energies and came to similar conclusions as to the magnitude and physical dimensions of the critical clusters for low-LET effects, with 100 eV or greater deposited in 3 nm spheres. This analysis was subsequently extended to heavy ions (Goodhead and Charlton 1985) where clusters of 340 eV or more in a cylindrical volume (10 nm diameter \times 5 nm length) correlated with the variation in the number of lethal lesions with LET. This analysis supported the theory that the critical lesion for high-LET biological effects correlated with a higher degree of clustering of energy deposition events with and associated higher biological effectiveness than that for low-LET effects. Again no assumptions were made about the

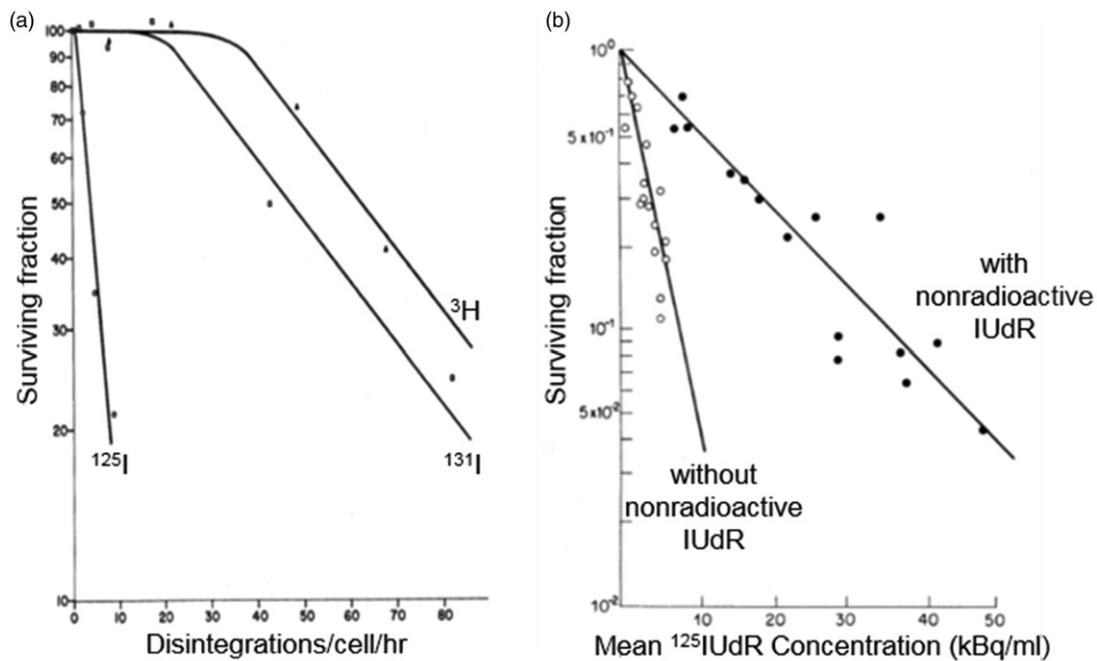


Figure 3. Auger emitters (which produce a cascade of very low energy, very short range electrons) become very effective when incorporated into DNA. (a) The DNA-bound short-ranged Auger-emitter ^{125}I UdR is significantly more effective at killing cells than the DNA-bound long-ranged beta-emitter ^{131}I UdR (Hofer and Hughes 1971, with permission), (b) The effectiveness of the ^{125}I UdR is significantly reduced if uptake of ^{125}I is blocked by the addition of nonradioactive IUdR (Kassis et al. 1987, with permission).

nature of the target, but the resulting target dimensions are consistent with the DNA structures, e.g. DNA helix (~ 2 nm diameter) and nucleosomes (~ 10 nm diameter \times ~ 6 nm length).

The importance of clustering of energy deposition events on the nanometer/sub-micron scale as a key factor in determining biological response also comes from a range of experimental data. For example, epithermal neutrons (25 keV) which mainly deposit energy through low energy recoil protons with mean track length of less than 100 nm, were found to be highly effective at inducing cell inactivation and chromosome aberrations (Morgan et al. 1988). Also C_K characteristic X-rays, which deposit energy via a low energy photoelectron with a range less than 7 nm, have an RBE of ~ 3 for a wide range of end-points (Goodhead and Nikjoo 1990; Hill 2004).

DNA as a target

The discrete nature of radiation tracks and their ability to initiate 'single-hit' responses have made them a useful probe of target size and sensitive volumes (Lea 1962). It was found in experiments on viruses, yeast, bacteria and mammalian cells that radiosensitivity is largely dependent on DNA content along with DNA repair capacity (e.g. Kaplan and Moses 1964; Thacker et al. 1989). Additionally, the early use of a proton microbeam (Zirkle and Bloom 1953) and alpha-particle emitting microneedles (Munro 1970) demonstrated that the main targets for radiation effect were within the cell nucleus. Strong evidence for DNA as an important radiation target also comes from the use of Auger emitters (which produce a cascade of very low energy, very short range electrons) which become very effective when incorporated into DNA

(Figure 3), but show a significant reduction in sensitivity when incorporation is blocked (Hofer and Hughes 1971; Kassis et al. 1987).

Clustered DNA damage

DNA damage is a constant and frequent challenge faced by all cells within the body, with the order of at least 50,000 endogenous DNA lesions being produced per cell per day, predominantly by reactive oxygen species (ROS) as a result of aerobic metabolism (De Bont and van Larebeke 2004; Swenberg et al. 2011). However, these lesions are constantly being repaired with high fidelity by a range of effective repair mechanisms that have evolved to maintain genome integrity. Interestingly, the number of DNA lesions produced following exposure to ionizing radiation is significantly less than the number of endogenous lesions, but these radiation induced lesions are extraordinarily more efficient for causing biological effects. For example, 1 Gy of γ -rays will result in approximately 850 pyrimidine lesions, 450 purine lesions, 1000 single-strand breaks (SSB) and 20–40 double-strand breaks; it is therefore surprising that a 3 Gy whole body dose has the potential to be fatal. The reason for this extraordinary difference is due to radiation track structure producing spatial and temporal correlation of these lesions.

Radiation induced DNA damage is produced either by direct ionization of its constituent atoms or indirectly through reactions with free radicals produced as a result of interactions in the surrounding water. Indirect DNA damage is dominated by hydroxyl radicals ($\cdot\text{OH}$) which are capable of producing either DNA base damage or strand breaks, however, the highly reactive environment within the cell limits their lifetime and therefore their diffusion distance to ~ 6 nm

(Roots and Okada 1975) thus restricting sites of damage to within a few nanometers of the path of the initiating radiation track. The passage of a single radiation track and the associated pattern of energy deposition events if it intersects with DNA (or passes within a few nanometers) can lead to a wide variety of molecular damage, including base damage, abasic sites, SSB and DNA-protein cross links. However, because ionizing radiation produces multiple energy deposition sites along the radiation track (correlated in time and space), it will frequently produce clustered damage sites, which consist of two or more lesions formed within one or two helical turns of DNA. A wide spectrum of clustered lesions, including DSB can be produced by a single radiation track. The spectrum of damage produced is critically dependent on radiation quality and this has been investigated over the years using Monte Carlo simulation of radiation tracks and modeling of the consequent DNA damage.

Charlton and Humm (1988) used Monte Carlo simulations of electron tracks in conjunction with a volume model of double stranded DNA in order to determine the spatial distribution of energy deposition along the DNA. Modeling of Auger electrons from DNA-incorporated ^{125}I resulted in excellent agreement with the experimental data of the spatial distribution of strand breaks. These calculations were subsequently extended to include indirect damage to DNA resulting from interactions of the radiation in the surrounding water and used to score DNA damage from low- and high-LET tracks. As a result, it soon became apparent that low- and high-LET radiations could produce wide spectra of clustered damages in DNA of increasing severity (e.g. Goodhead 1994; Nikjoo et al. 1997; Nikjoo et al. 2001, 2002; Watanabe et al. 2015). These studies indicate that even for low-LET radiation approximately 20–50% of the DSB produced are in fact complex DSB (with extra strand-breaks and/or associated base damage; Nikjoo et al. 2002; Goodhead 2006). The frequency and complexity of these complex DSB increase with increasing ionization density (LET), with >90% of DSB being complex for high-LET α -particles (Nikjoo et al. 2001). It is important to note that these clustered lesions arise from a single track, a dose of $>10^4$ Gy would be required for a second independent radiation track having a reasonable chance of contributing to the local complexity at the site (Nikjoo and Goodhead 1991). These calculations and associated experiments investigating the variation of the yields of SSB and DSB at different OH scavenging conditions demonstrate the important contribution of indirect damage to the cluster and the need to appropriately model the chemistry of water radiolysis products produced following irradiation taking into account the high scavenging conditions within the nucleus (Fulford et al. 2001). These approaches have been developed over the years to cover a wide range of radiation qualities including light ions relevant for radiotherapy (e.g. Baiocco et al. 2016; Nikjoo et al. 2016; Friedland et al. 2017).

Cells have a number of DNA repair pathways that can deal with a range of DNA damage, therefore, an important factor in determining the ultimate fate of the cell is the initiated biological response and the consequence of any attempt to repair the damage. The increased complexity

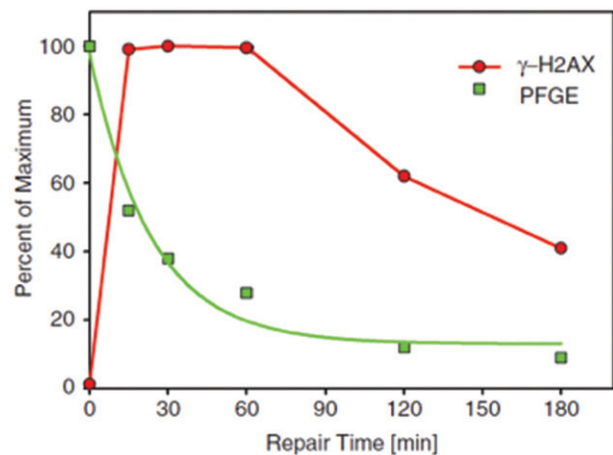


Figure 4. Comparison of DSB repair kinetics measured using Pulse-Field Gel Electrophoresis (PFGE) with a typical time response observed for γ H2AX foci measured using immunofluorescence (Kinner et al. 2008, with permission).

corresponds to a decreasing probability of faithful repair and therefore greater biological effectiveness (Goodhead et al. 1993). This is illustrated in experimental studies showing an increase in the proportion of slow-repairing DSB with high LET radiations (Jenner et al. 1993; Asaithamby et al. 2008) which are more likely to be mutagenic or lethal (Dobbs et al. 2008). The variation in complexity of DSB is also reflected in the variability in biological efficiency per DSB produced as a function of radiation quality; with even the cell lethality of X-ray produced DSB estimated to be 4–40 times more effective than simple DSB produced by hydrogen peroxide (Prise et al. 1994). For low-LET radiation, it is expected that the damage will be dominated by fairly simple clustered damage including simple DSB, as a result the biological response can therefore be easily and substantially modified, for example, by the presence of oxygen ('fixing' individual lesions) or modifying the efficiency of the various DNA repair pathways. Modification of the response is limited for high-LET radiation, as the complex clustered lesions produced are already difficult to repair even with fully functioning repair pathways and in the absence of oxygen. A major area of current research is modeling the biological response to DNA damage and the resulting repair (e.g. Cucinotta et al. 2008; Friedland et al. 2010; Taleei and Nikjoo 2013; Li et al. 2014). For example, Taleei and Nikjoo (2013) and Taleei et al. (2013, 2015) have developed a biochemical kinetic model for non-homogeneous end joining (NHEJ) repair of simple and complex DSB (determined from track structure calculations) with the results confirming that DSB complexity is a potential explanation for the slow component of repair and capable of predicting realistic repair kinetics for a range of radiation qualities.

Currently, DSB induction and repair are commonly experimentally studied using γ H2AX or 53BP1 antibodies in conjunction with immunofluorescent microscopy or flow cytometry as a marker of sites of DSB. While this has advantages over older techniques in the ease of use and greater sensitivity, care must be taken with respect to inferring actual DSB yields and repair kinetics (Figure 4). While previous studies using pulse field gel electrophoresis (PFGE) demonstrate the majority of DSB produced by low-LET radiation are

quickly repaired with a half time of ~ 20 min; many laboratories report an increase in γ H2AX foci with time reaching a maximum ~ 30 – 60 min post exposure, at times when the majority of DSB are expected to have already been repaired (Gulston et al. 2004; Kinner et al. 2008). The subsequent rate of loss of foci are also slower than the repair kinetics obtained using PFGE. Also, in addition to radiation induced DSB, γ H2AX can also be formed at the site of stalled replication forks (Rothkamm et al. 2015) and may also be generated during DNA transcription activity (Dickey et al. 2012).

Although many studies focus on the induction and repair of DSB, ionizing radiation can also efficiently produce non-DSB clustered lesions, these consist of two or more lesions on the same or opposite strands. These make up a significant component of DNA damage produced by ionizing radiation, with the frequency and spectra again dependent on radiation quality (Watanabe et al. 2015). While isolated lesions are repaired quickly with high fidelity, the rate of repair of these non-DSB clustered lesions is typically significantly impaired as a result of the associated neighboring damage sites and dependent on the type, number, separation and orientation of these lesions (Tsao et al. 2007; Magnander et al. 2010; Eccles et al. 2011). Due to the increased lifetime of these lesions, there is an increased probability of them resulting in a stalled replication fork and potentially a replication induced DSB, which may be complex by virtue of additional lesions close by (Gulston et al. 2002 and Malyarchuk et al. 2009). These clustered lesions have been shown to result in enhanced mutation frequencies experimentally demonstrated in bacteria, yeast and mammalian cells (Georgakilas et al. 2013). The frequency of induction of non-DSB clustered lesions for low-LET X-ray and γ -ray exposures is at least 4–8 times greater than prompt DSB (Eccles et al. 2011). So, while at high doses it is likely that the DSB will dominate the response; at low doses of low-LET radiation there will be many more cells with non-DSB clustered lesions and no DSB. It has been proposed that the delayed repair of these non-DSB clusters if persisting to replication, can either lead to the mis-incorporation of bases or the formation of replication induced DSB which could ultimately lead to chromosome aberrations, genetic instability and tumorigenesis (Eccles et al. 2011). It also must be remembered that clustered damage is not just confined to DNA but other molecular structures within a cell, however, the effects are often limited due to the existence of many copies.

The frequency and spectrum of clustered DNA damage is not only dependent on LET, but also on particle type. It is the 3D distribution of energy deposition that is important, while LET is a 1D quantity. It is well known that different charged particles of the same LET can differ in their biological effectiveness for given dose, typically decreasing with increasing atomic number. This is due to a reduction in local energy density along the track associated with the greater range of the delta-ray electrons produced. For example, the maximum range of delta-rays for a 1.8 MeV α -particle is the order of $0.1 \mu\text{m}$ (with $\sim 90\%$ of energy deposited within ~ 10 nm), the delta-rays from a 1 GeV amu^{-1} Fe ion of similar LET ($150 \text{ keV } \mu\text{m}^{-1}$) are capable of producing low-LET damage up to a several millimeters away in neighboring cells.

Although the variation in RBE as a function of energy/LET is often discussed with respect to neutrons and ions (such as protons and α -particles), there is also significant experimental evidence demonstrating an increasing RBE with decreasing photon (i.e. X- and γ -ray) energy (ICRP 2003; Hill 2004). These photons interact producing a broad spectra of electron energies. Calculations show that for ^{60}Co γ -rays $\sim 33\%$ of absorbed dose is deposited via low energy electrons track ends (0.1–5 keV), with this contribution increasing to $\sim 49\%$ for 220 kV X-rays and further dominates at lower energies (Nikjoo and Goodhead 1991). Studies using ultrasoft X-rays have demonstrated that these low energy track ends have significant enhanced RBEs for a range of biological endpoints including DSB induction (even for the very short electron tracks (< 7 nm) produced by C_K X-rays). This results from an increase in clustering of ionizations and therefore DNA damage over nanometer distances, leading to an increase in the absolute number of DSB and complexity of resulting lesions. It has been proposed that these low-energy electron tracks are the biologically relevant component of all low-LET radiation (Goodhead and Nikjoo 1990; Botchway et al. 1997). The variation in biological effectiveness with photon energy does mean that the RBE values obtained for particle irradiation are critically dependent on the reference source used and it is therefore important that this is adequately described (including any filtration used) to enable appropriate comparison with other data. Also, although ICRP have assigned a radiation weighting factor, w_R of 1 for all low-LET radiation for implementation of radiation protection legislation, they do acknowledge that this may not be appropriate for specific risk calculations (ICRP 2007).

The micrometer/nuclear scale

DNA is organized within the cell nucleus in the form of chromosomes (Figure 1). DNA wraps around histones forming nucleosomes (~ 10 nm diameter), which themselves coil and stack together in chromatin fibers (~ 30 nm), these are organized into loops and ultimately occupy discrete domains within the cell nucleus. High-LET particles typically deposit energy along discrete tracks. As a result of the high density of ionization events along the tracks, as illustrated in Figure 1 they can produce correlated DNA damage across these higher orders of DNA packing (e.g. nucleosome, chromatin fiber/loops and even across the nucleus). This has been demonstrated theoretically and experimentally, with increasing deviation from random breakage of DNA with increasing LET and associated enhancements of DNA fragments of < 300 kbp (Friedland et al. 2008), with a peak in short DNA fragments of the order of ~ 80 bp correlating to DNA wound around histones, with additional smaller peaks for larger fragments out to a shoulder at 450 bp (Rydberg et al. 1998). It is important to note that most assays are not capable of resolving these closely spaced correlated DSB and as a result the experimental yield of DSB will typically be underestimated with increasing LET (Prise et al. 2001). The ultimate relevance of these correlated breaks will depend on the ability of the cell to repair the individual damage sites,

however, this may also be compromised by illegitimate repair between other sites of damage nearby.

The ability of ionizing radiation to induce genetic effects was demonstrated by Muller (1927) who showed that X-rays were able to induce phenotypic mutations in *Drosophila* indistinguishable from those produced spontaneously. The ability of ionizing radiation to induce structural chromosome aberrations was demonstrated in Sax (1938); this and many of the early experiments (reviewed by Lea, 1962) formed the basis of classical radiation biology. However, with the advent of fluorescent in situ hybridization (FISH) cytogenetics techniques and subsequently combinatorial multi-fluor techniques (e.g. SKY and mFISH) for karyotyping individual chromosome pairs, it soon became clear that some of the observed chromosomal rearrangements were often far more complex than previously thought involving multiple chromosomes and breaks. Although the SKY and mFISH techniques are good at identifying inter-chromosome exchanges, they are poor at identifying intra-chromosomal rearrangements; this has started to be addressed to some degree with the development of techniques such as multicolor banding (mBAND) (Chudoba et al. 1999) and the use of strand-specific directional probe in conjunction with single-stranded hybridization (Ray et al. 2013).

There are three main pathways that have been proposed to explain these chromosomal rearrangements. The breakage-and-reunion pathway, where the two free ends associated with one radiation induced break may separate and ultimately misrejoin with the free ends of surrounding radiation induced breaks. A second possibility is Revell's exchange theory, where radiation induces unstable lesions which do not form free ends that can separate; however, if two of these lesions come together they can initiate an exchange process. A third potential pathway that has been put forward is the molecular (1-hit) theory, where a single radiation induced lesion initiates an exchange with undamaged DNA, with the second break being created enzymatically in a process similar to homologous misrepair. In part as a result of the complexity of aberrations observed and associated dose response, the breakage-and-reunion theory is the current favored model (although it is always possible that more than one mechanism may be in operation). However, regardless of the pathway, the resulting chromosome aberration spectra is dependent on spatial distribution of ionizing radiation induced DNA lesions, the chromosome geometry and their arrangement within the nucleus.

In addition to the difference in energy deposition on the sub-micron scale, there are also significant differences on the micrometer/cellular scale between different radiation qualities which can play an important role in determining the biological response. For example, assuming an 8 μm diameter spherical cell, 1 Gy of low-LET radiation (e.g. γ -rays) corresponds to approximately 1000 electron tracks depositing energy essentially randomly distributed across the volume. In contrast, 1 Gy of high-LET α -particle corresponds to ~ 2 –4 tracks traversing the cell, with highly heterogeneous energy deposition along these straight, narrow densely ionizing tracks (due to short range of the delta-electrons, the majority of energy is deposited $\ll 0.1 \mu\text{m}$ from the track). Therefore,

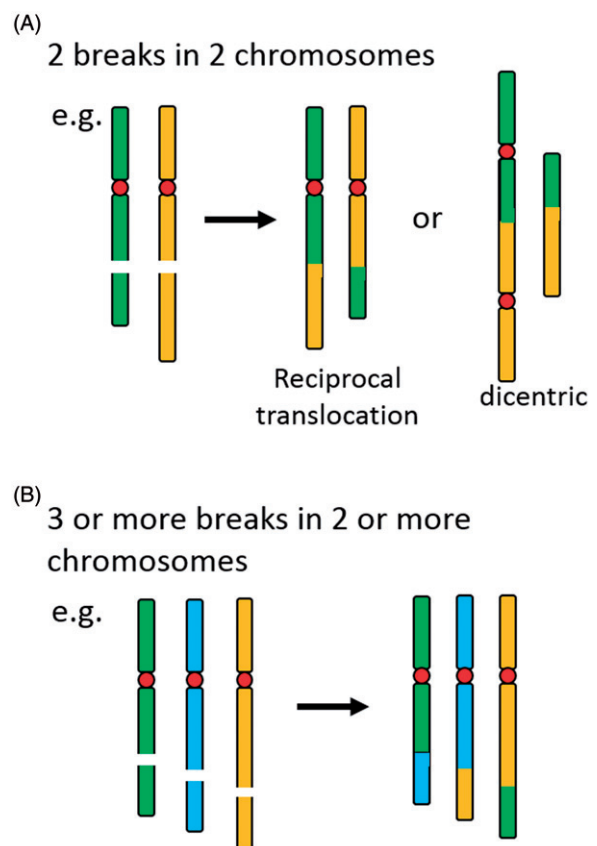


Figure 5. Examples of (A) simple chromosome exchanges (involving two breaks in two chromosomes) and (B) a complex exchanges (involving three or more breaks in two or more chromosomes). Note that the rearrangements for the reciprocal translocation and the example complex exchange shown here would be difficult to discern using traditional staining techniques.

the resulting 20–40 DSB produced by low-LET radiation will be essentially homogeneously distributed across the nucleus and associated chromosomes. While for α -particles, the relatively similar number of DSB produced will be highly correlated along the narrow tracks as it traverses the nucleus, not only within individual chromosomes but also between adjacent chromosome territories traversed (Figure 1). As a result of the correlation of these breaks in time and space increasing the probability of genetic rearrangements between these sites, complex chromosome aberrations (requiring three or more breaks in two or more chromosomes; see Figure 5) are characteristically produced by high-LET particles (Anderson et al. 2002). In contrast to the production of mainly simple chromosome aberrations (maximum of two breaks in two chromosomes, see Figure 5) observed for low doses of X-rays, a wide spectrum of aberrations result from a single α -particle traversal (for peripheral blood lymphocytes an average complex will typically involve six breaks in four chromosomes). However, the variation in nuclear geometry with respect to this track will also influence the resulting yields and complexity of aberrations (Durante et al. 2010). The likelihood of aberrations being classified as complex, as opposed to simple, was found to increase with decreasing α -particle energy, as a result of increasing ionization density (LET) and the associated increase in frequency and complexity of DSB along the track (Anderson et al. 2007). The importance of the

spatial distribution of dose deposition and therefore DNA breaks across the nucleus on the micrometer scale in determining biological response has recently been demonstrated recently using patterned delivery of 20 MeV protons (Schmid et al. 2012). It was observed that the RBE for micronuclei and dicentric chromosomes were significantly raised when the protons were focused to submicron spots delivered in a $5.4 \times 5.4 \mu\text{m}^2$ matrix compared to the same dose delivered using a quasi-homogeneous $1 \times 1 \mu\text{m}^2$ matrix distribution. The probability of illegitimate recombination between breaks increasing when they are concentrated in the sub-micrometer spots.

Visible chromosome aberrations potentially just represent the tip of the iceberg of what is really going on. As well as the difficulty in identifying intra-chromosome exchanges, one of the main limitations of these cytogenetic techniques described above is that the resolution is typically limited to the order of 1–10 Mb, which therefore limits their ability to fully reveal the complexity of exchanges, potentially missing small deletions or insertions (Cornforth 2006). In addition, the DNA damage studies described above have already highlighted that high-LET radiation can produce correlated sites of DNA damage smaller than this limit. One approach that has been used to address what is happening at the gene level has been to sequence radiation induced gene mutation events in the progeny of surviving cells. Interestingly studies have shown that the vast majority of radiation induced *HPRT* mutations are not large enough to be detectable using FISH techniques. Larger scale deletions were typically reported for high-LET radiation compared to X-/ γ -ray or spontaneous mutations, with some studies showing multiple deletion sites (Schwartz et al. 1994; Zhu et al. 1996; Schmidt and Kiefer 1998; Singleton et al. 2002; Rothkamm et al. 2008). However, it is important to note that experimental studies just looking at the pattern of loss of unaltered PCR products for exons often underestimated the complexity of rearrangements (Rothkamm et al. 2008). Also, when detailed sequencing was performed on α -particle induced mutations that initially appeared to be simple deletions, some of these were found to be far more complicated showing combinations of deletions, insertions and inversion of DNA sequence (Singleton et al. 2002). It is far from clear what the mechanisms underlying these complex rearrangements are.

In addition to experimental studies, Monte Carlo models have been developed that now incorporate the various orders of packing from DNA wrapped around nucleosomes, to chromosome fiber and loops and with individual chromosomes occupying discrete domains within the nucleus. In addition to predicating lesion complexity at individual sites of DNA damage, they can also determine the spatial distribution of initial breaks across individual chromosomes, along with the distribution across all traversed chromosomes in order to model chromosome aberration formation (Friedland et al. 2008; Ponomarev et al. 2012; Friedland and Kundrat 2013). This line of research would greatly benefit from advances in experimental techniques to help reveal the full complexity of the genetic rearrangements, associated kinetics and critically test the models.

The millimeter/tissue scale

Most radiobiology experiments are performed at relatively high doses where cells are traversed by multiple tracks. While this may be applicable to therapeutic doses of ionizing radiation, where typically a dose of 2 Gy per fraction is delivered to the tumor, most human exposures correspond to single track interacting with the cell. Environmental levels of exposure to low-LET radiation typically corresponds to approximately 1 interacting electron track per cell nucleus per year, and on average this corresponds to a dose to the cell nucleus of the order of 1 mGy. While typical environmental radon exposures to high-LET alpha-particles correspond to approximately 0.002–0.009 alpha-track traversals per year of a cell nuclei in the bronchial epithelium (National Research Council 2006); while the vast majority of cells are not traversed, those that are receive a substantial local dose of up to ~ 0.5 Gy.

Historically, all biological effects of ionizing radiation were believed to be a due to 'targeted' effects, with initial DNA damage produced at sites where the radiation tracks interact and directly leading to permanent modifications of DNA and chromosomes. While these effects are biologically important, it has become clear in recent years that ionizing radiation can also initiate processes in which biological effects can be observed in locations removed in space (e.g. effect observed beyond the irradiated cell), and in some circumstances in time (e.g. effect observed in the progeny of irradiated cell), from the initial damage. These effects are commonly known as 'non-targeted' effects and include (among other effects) bystander effects and radiation induced genomic instability which can produce a range of endpoints including, induction of mutation and chromosome aberrations, changes in gene expression changes and cell killing. These non-targeted effects and associated mechanisms have been reviewed in detail over the years in the literature (Morgan 2003a, 2003b; Morgan and Sowa 2007; Hei et al. 2008; Prise and O'Sullivan 2009), however, the 'targets' for initiating these responses have still to be identified. In brief, bystander effects relate to responses induced or modulated in non-irradiated cells as a result of the ionizing radiation perturbing stress-inducible signals involving multiple signaling pathways which form part of the ongoing natural communication that exists between cells within tissues (Hei et al. 2008). This can ultimately lead to persistent inflammatory response which has been linked to promoting tumor progression (Morgan and Sowa 2007; Hanahan and Weinberg 2011). While induced genomic instability refers to when these effects are observed in the progeny of cells many generations after the parental cells have been irradiated (Kadhim et al. 1992). These are heterogeneous arising non-clonally within the decedent population, occur at a higher frequency than can be explained by a specific gene mutation and have also been observed in bystander cells. Genomic instability is also regarded as enabling characteristic for cancer with its enhanced mutability producing the genetic alterations that can drive tumor progression (Hanahan and Weinberg 2011). Interestingly, the dose response for these effects are such that they are typically observed to be induced and then to plateau at very low

doses, similar to those associated with diagnostic X-ray exposures (Portess et al. 2007; Prise and O'Sullivan 2009). While it is likely that direct targeted effects of radiation will dominate at high doses associated with radiotherapy, these non-targeted effects have the potential to play a role in modulating the frequency of a range of biological effects following low dose exposure to radiation, especially for high-LET particles where the traversed cell will receive substantial dose.

Summary

Understanding the mechanisms underlying radiation induced biological effects is important for not only understanding the risk of exposure, but also optimization of radiotherapy. Historically, the field of radiation biology has been developed as a result of interdisciplinary research between physicists, chemists, biologists, clinicians, mathematicians and more recently computer scientists. Maintaining this interdisciplinary approach will be key for future developments, along with a continued emphasis on careful quantification of experimental results and the development of mathematical/computer models. However, these models and their predictions are only as good as the input data and assumptions made and therefore must be critically tested and carefully benchmarked against experimental data (taking into account any limitations of the experimental data). Likewise, experimentalists should be looking to design experiments to critically test these models and associated assumptions, as it is important to identify the limitations of models and situations where they may not be valid. The historical research presented in this paper just covers one small field of radiation biology that has benefited from this interdisciplinary and analytical approach.

For ionizing radiation, it is clear that the spatial and temporal distribution of energy deposition along the resulting radiation tracks is key in influencing biological outcomes and explain the variation in biological effectiveness for different qualities of radiation. However, this is a multifaceted problem covering many scales (Figure 1), from DNA damage produced on the nanometer scale, correlation of this damage on the micrometer scale and even the millimeter scale and beyond in tissues due to the influence of perturbing intercellular signaling. Clustering of energy deposition on the nanometer scale has been shown to play a critical role in determining biological response, producing not just simple isolated lesions but also clustered lesions (including complex DSB) that are more difficult to repair. The frequency and complexity of these clustered damage sites is typically found to increase with increasing LET. Although the focus is often on DSB and complex DSB, non-DSB clustered lesions are also likely to play a role. However these sites of DNA damage should not be considered in isolation. In order to fully understanding the consequences, it is important to look at the relative distribution of these lesions over larger dimensions along the radiation track, up to the micrometer scale. Correlation of events can result in complex gene mutations and complex chromosome rearrangements with the frequency and spectrum of the resulting rearrangements

critically dependent on the spatial and temporal distribution of break sites and therefore the radiation track. Due to limitations in the techniques used to identify these rearrangements it is likely that the full complexity of the genetic rearrangements that occur has yet to be revealed. The mechanisms associated with these rearrangements are still not fully understood. While classical target theory and associated DNA damage are likely to dominate cellular response at high dose, non-targeted effects may well be important for low dose exposure, especially for high-LET particle exposure (e.g. α -particles), where the track structure is such that the dose to the traversed cell will be relatively large producing significant damage, but this will be surrounded by many non-traversed cells which can be influenced by perturbing the homeostasis of intercellular signaling and associated changes in oxidative stress.

Research into the biological effects of ionizing radiation has a long history spanning over a century following the discovery of X-rays by Roentgen in 1895 and radioactivity by Becquerel in 1896. This has resulted in a significant amount of detailed research during this period in a range of *in vitro*, *in vivo*, *in silico* and other model systems, which contain useful data and lessons which can still cast light on current research and guide future studies. There is often a tendency to just concentrate on current studies and new techniques; however, all techniques have their advantages and limitations. If it is new, it does not necessarily mean that it is the best data and because it is old does not mean it is wrong or irrelevant. So, let us build on past knowledge to effectively explore the future.

Acknowledgements

The author would like to thank Dudley Goodhead for his helpful comments.

Disclosure statement

The author reports no conflicts of interest.

Funding

Funding provided by the Medical Research Council Strategic Partnership Funding (MC-PC-12004) for the CRUK/MRC Oxford Institute for Radiation Oncology is also gratefully acknowledged.

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