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Radiation-induced unrepairable DSBs: their role in the late effects of radiation and possible applications to biodosimetry

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ABSTRACT

Although the vast majority of DNA damage induced by radiation exposure disappears rapidly, some lesions remain in the cell nucleus in very small quantities for days to months. These lesions may cause a considerable threat to an organism and include certain types of DNA double-strand breaks (DSBs) called 'unrepairable DSBs'. Unrepairable DSBs are thought to cause persistent malfunctioning of cells and tissues or cause late effects of radiation, especially the induction of delayed cell death, mutation, senescence, or carcinogenesis. Moreover, the measurement of unrepairable DSBs could potentially be used for retrospective biodosimetry or for identifying individuals at greater risk for developing the adverse effects associated with radiotherapy or chemotherapy. This review summarizes the concept of unrepairable DSBs in the context of persistent repair foci formed at DSBs.

Keywords: radiation; unrepairable DSBs; gamma H2AX; senescence; biodosimetry

INTRODUCTION

Among the genome damage induced by ionizing radiation, DNA double-strand breaks (DSBs) represent the most biologically deleterious type of lesion. To tackle these potentially lethally damaging lesions, cells have evolved orchestrated and conserved mechanisms known collectively as the DNA damage response (DDR) [1–4]; the DDR coordinates cellular DSB repair activities immediately after the damage is detected [4]. Indeed, within seconds, DSB repair proteins start to accumulate at the site of DSBs, and the DDR directs the cells to repair the breaks, undergo apoptosis, or become senescent [3]. DSB repair is a quick and efficient process whereby broken ends are rejoined. Using traditional and biochemical DNA size analyses [5–7] coupled with immunocytochemical staining [8–10], the kinetics of DSB repair have been shown to occur in two phases: a fast phase lasting up to a few hours, with a half-life of 30 min to 1 h, followed by a slower phase that may persist for a long time. A few persistent DSBs remain into the next day, and some of these DSBs persist for days, months, or even years [10–13]. These DSBs are more difficult to repair or remain unrepaired DSBs. Alternatively, these persistent DSBs are termed residual DSBs and are retained in the damaged cells unless the cells die and slip away [10, 14]. The number of unrepairable DSBs in a cell is measured by detecting and counting the number of persistent repair foci, which are composed of multiple proteins that accumulate at DSBs. Specifically, it is suggested that individual γH2AX or 53BP1 foci represent a single DSB with a ratio of 1:1 [8, 15, 16]; hence, very few repair foci out of the many that form immediately after radiation exposure remain and become persistent in the cell nucleus. In this review, I summarize the observations obtained from the exposure of quiescent normal human cells to radiation, incorporate these observations into a discussion of the literature, and then discuss the biological implications of unrepairable DSBs with respect to the effects of radiation on cells and tissues. I also discuss the possible application of unrepairable DSB measurement for retrospective biodosimetry.

GENERATION OF UNREPAIRABLE DSBS AND PERSISTENT REPAIR FOCI

Radiation-induced DSBs are first detected by the MRE11/Rad50/NBS1 (MRN) complex and the Ku70/Ku80 heterodimer [1, 3]. Accordingly, the DDR initiates many possible mediators and effectors to potentiate distinct signals within a cell. The MRN complex promotes the binding of a phosphorylated form of ATM and the phosphorylation of H2AX. The MRN complex also promotes the

binding of MDC1 and a group of ubiquitination proteins (RNF8 and RNF168) and their effectors at the DSBs, thereby forming the fundamental structures of repair foci. Repair foci are a cluster of many kinds of proteins. They have different components depending on whether the cells are undergoing the NHEJ pathway (including microhomology-mediated NHEJ) or entering S phase, during which the cells undergo HR-mediated repair. In fact, in exponentially growing normal human fibroblasts, 100% of cell nuclei have many γH2AX/53BP1 foci after radiation exposure, whereas only 30% of those exhibit BRCA1-positive foci. The repair foci containing BRCA1 are specifically formed for recombination repair in S phase (Fig. 1). This observation indicates that the accumulation of BRCA1 protein depends on cell progression through S phase. In somatic cells in living organisms, most of the cells are in quiescent (Go) phase; they are not dividing and are instead terminally differentiated, functioning to preserve their specified phenotype and exhibiting very long lifespans, up to years or decades. To create a model system for this *in vivo* cell status, we have adopted quiescent cultures of normal human diploid fibroblasts (NHDFs) in which the cells are maintained in MEM + 0.1% FCS. In this condition, the cells are permanently growth arrested (Go state) and can survive as long as weekly medium changes are maintained (any time after quiescence, cells can reenter the cell cycle in the presence of medium containing 10% FCS, which provides growth stimulation).

Analysis of the DSB repair kinetics of quiescent NHDFs showed that 95% of the initial DSBs disappeared within a couple of days after radiation exposure, followed by a very slow decline to ~1% DSBs remaining after 2 weeks. These DSBs persisted in the cell nucleus as long as the culture continued (up to 1 year in our study) [10], thus we designated them 'unrepaired DSBs'. The formation and resolution of γH2AX/53BP1 foci have been extensively studied *in vitro* [8, 17–20], and the kinetics have been shown to be biphasic [10–12, 21], which matches well with the kinetics of DSB repair [5]. Although the persistence of repair foci could arguably reflect insufficient H2AX dephosphorylation [22], it has been readily accepted that the foci represent unrepaired DSBs because the foci also include 53BP1 and all other previously reported DDR proteins. Moreover, the phosphorylated form of DNA-PKcs (ser-2056), which marks the initial recognition of a DSB, was also detected in the foci [10]. Treatment of cells with the ATM inhibitor KU55933 completely abolished the persistent foci, and the foci recovered at the DSB sites when the inhibitor was chased off. This effect was observed in cultures even 6 months post exposure to radiation. Treatment with the polyubiquitination inhibitor MG132 also produced the same effects. These results indicate that there is a continuous turnover of foci components at the unrepaired DSBs, which persists for a long time after the exposure.

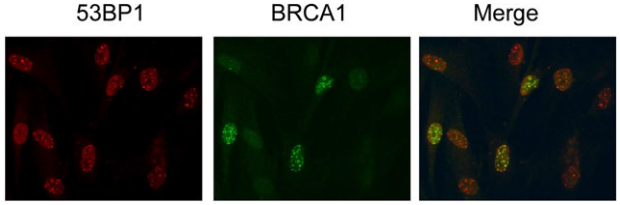

Fig. 1. Staining of exponentially growing NHDFs immediately after radiation exposure (1 Gy/1 h) with antibodies against 53BP1 and BRCA1.

Therefore, what is the function of the slow to very slow type of DSB repair that follows the fast phase? Is it truly a process of very slow repair, does this process establish unrepaired DSBs, or is it a mixture of both processes? At present, we do not know the biochemical difference between repairable and unrepairable DSB foci; however, it is almost certain that the size of the foci grows larger over time [10, 23–26]. We measured the foci enlargement over time and found that the unrepaired foci could be sorted from the small foci of the fast phase that would eventually disappear upon the completion of repair. The repairable foci displayed a normal size distribution immediately after radiation exposure, whereas after 1 or 2 months the unrepaired DSB foci formed a distinct distribution of foci larger in diameter and could be separately counted (Fig. 2).

PROSPECTIVE APPLICATIONS OF UNREPAIRABLE DSBS TO BIODOSIMETRY

We know that the effects of radiation stem from unrepairable damage and not from the repair process of repairable damage. It has been argued that the radiosensitivity of an individual cell can be attributed to the presence of unrepairable DSBs [27–29]. These unrepairable DSBs are responsible for the late effects of radiation, i.e. changes in the aging process, late onset mutations, and cancer. It is also argued that the measurement of unrepairable DSBs could be applied to radiation dosimetry on previously irradiated cells and tissues [30–33].

By counting the number of large-sized foci remaining 1 month after irradiation, we found that there was a linear relationship between the radiation dose and the number of unrepaired DSBs. Assuming that one unrepairable DSB is one lethal hit, then the actual average one-hit dose is 'D_o', which is the dose required for 37% cell survival in classic 'radiation hit theory'. A similar idea enables the calculation of the average two-hit dose required for 13.5% cell survival. Then, from the dose–response curves of the formation of unrepairable DSBs we made hypothetical survival

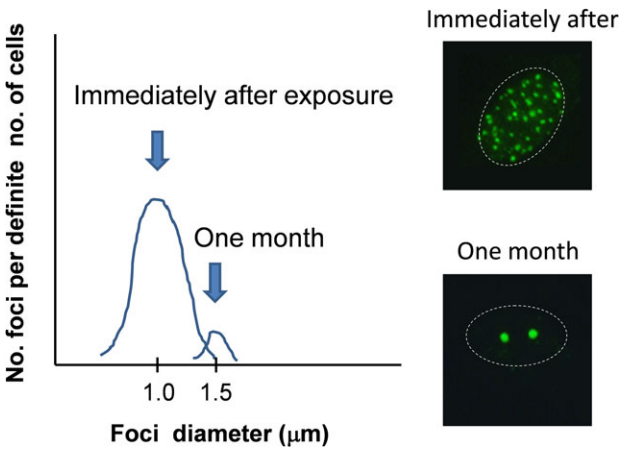

Fig. 2. Images of the size distribution of repair foci immediately after or a long time after radiation exposure. One month after exposure, transient/repairable DSB foci disappeared, and persistent DSB foci remained in the nucleus.

curves, which agreed well with actual cell survival phenotypes. The concept that unrepairable DSBs limit the radiosensitivity of cells has been validated by other studies [28, 29]. In fact, Menegakis *et al.* have also attempted to calculate hypothetical cell survival [34].

Based on observations that the number of unrepairable DSBs accumulates with repeated exposure [10, 20], retrospective radiation biodosimetry has been proposed [10, 20, 35, 36]. By measuring the number of unrepaired DSB foci per unit area of mouse skin, Bhogal *et al.* reported the possibility of biodosimetry [28]. In minipig, skin biopsies taken 70 days after a 50 Gy exposure showed typical unrepaired DSB foci patterns. Accordingly, minipig [37] and macaque models [38] for persistent DSB-mediated biodosimetry have been reported. Cells from plucked hair appeared to be especially useful [32]. In mouse spinal cord, the persistent foci were detectable 1 year after the exposure [39]. Overall, these reports may offer a new avenue for the use of unrepairable DSBs in radiation biodosimetry. We also observed the appearance of large repair foci one month after mouse pancreas was irradiated with 6 Gy. Similar observations have been reported in human buccal cells [40] and mouse germ cells [39, 41]. Moreover, increases in the number of unrepairable DSBs have been associated with aging in many studies [20, 36, 42–44].

Some pediatric cancer patients display increased unrepairable DSB production levels in their peripheral blood lymphocytes. These patients might represent groups at higher risk during radiation exposure [45]. A group of patients who demonstrated an excessive response to radiotherapy, which was assessed by normal tissue toxicity, exhibited increased levels of persistent repair foci [46]. The levels of persistent repair foci per cell in lymphocytes were examined after radiotherapy in breast cancer patients [29, 47]. This calculation will enable the fine-tuning of radiation doses for improved cancer treatments. In head and neck cancer cases, prescreening the levels of persistent foci in 2 Gy irradiated lymphocytes was applied to reduce the side effects of radiotherapy [48]. Thus, persistent repair foci are a useful measure for the detection and evaluation of previously irradiated cells.

However, it has been reported that repair kinetics and the formation of persistent repair foci vary extensively among mouse tissues [49, 50]. Therefore, tissue differences should be considered when evaluating unrepairable DSBs for biodosimetry.

THE REAL IDENTITY OF UNREPAIRABLE DSBS: ARE THEY TELOMERE DSBS OR ARE THEY LOCATED INSIDE THE CHROMOSOME?

Although the chemical structures of unrepairable DSBs have not yet been precisely determined, two types of 'complex' lesions can be postulated: those with non-ligatable termini due to crosslinks between bases and sugars (dirty DSBs) and those with damaged sites in which multiple DSBs/SSBs and/or base damage arise in close proximity (clustered damage) [51]. The complexity of the damage structure is evident by the slow rejoining kinetics of DSBs induced by high-LET radiation [52]. It is apparent that the damage induced by radiation is extremely heterogeneous and that damage occurs randomly throughout the chromosome.

Unrepaired DSBs induce permanent cell growth arrest or death. This fact is especially true in non-apoptotic terminally differentiated cells, which in general have very long lifespans. In these cells, cellular senescence is induced (radiation-induced senescence). It has been shown that many adult survivors of juvenile cancer develop symptoms of premature aging later in life [53], which may be related to the unrepaired DSBs that were induced after radiotherapy in normal tissues adjacent to the tumor. Their symptoms include advanced frailty with increased risk for heart failure, severe cognitive decline, coronary heart disease, and secondary neoplasms [53–55]. Meanwhile, neuronal stem cells bearing unrepairable DSBs undergo premature senescence or terminal differentiation to become astroglial cells [56]. This process also reduces stem cell number, leading to a premature aging of neuronal systems. Likewise, after radiation exposure, melanocyte stem cells in hair bulge undergo terminal differentiation in their niches, producing gray hairs [57].

Telomere-driven replicative senescence has been widely accepted as a primary mechanism of organismal aging and cellular senescence. Under this theory, eroded chromosome ends continuously activate the DDR, thereby permanently inducing growth arrest and senescence. Similarly, it has been hypothesized that radiation-induced unrepairable DSBs eventually accumulate in chromosome ends, which establishes senescence independent of the cell's telomere length [14, 35, 36, 58]. This idea is based on the specific structure of chromosome ends, known as Shelterin, which protect chromosomes from improper recombination and degradation. Indeed, TRF2 and RAP1, the components of Shelterin, were shown to inhibit DSB repair throughout the entire telomere [35, 59–61], not only at the very ends. Therefore, radiation-induced DSBs near the chromosome ends would be preferentially protected from repair systems, even though radiation exposure induces DSBs randomly throughout the chromosome. Such a mechanism may eventually provoke continuous DDRs, leading to cellular senescence in cells bearing radiation-induced unrepairable DSBs (Fig. 3A).

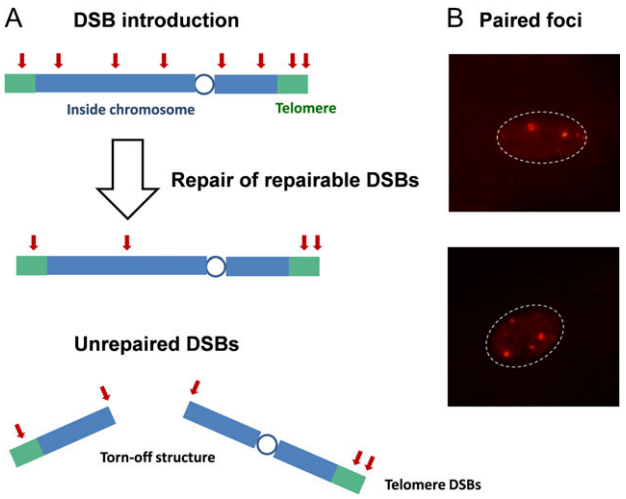

Fig. 3. (A) Possible mechanisms for the generation of unrepaired DSBs. Although radiation exposure induces DSBs randomly in the chromosome, those occurring proximal to the telomere or unrepairable DSBs inside the chromosome eventually become persistent. (B) Persistent repair foci tend to make pairs. In the lower photo, two pairs of foci of different sizes can be seen.

This idea has been confirmed via confocal microscope observations and telomere ChIP experiments [36]. Thus, the telomere hypothesis suggests that the location, not the chemical structure, is important for the establishment of unrepairable DSBs. This hypothesis may explain the radiation-induced senescence of young cells *in vitro* and non-dividing quiescent cells *in vivo*. Both of these cell types have long telomeres and no chance of undergoing replicative senescence. Assuming that animals carrying longer telomere sequences have a wider target for the creation of unrepairable DSBs, telomere length may have a negative effect on lifespan. Indeed, inverse correlations between telomere length and lifespan have been reported in different animal species [62].

However, other mechanisms of radiation-induced senescence may exist. Most nuclei of 6 Gy-irradiated and 1-month-cultured young quiescent cells carried a few typical, large γH2AX/53BP1 foci, as mentioned above. Careful observations have revealed that these foci often appear in pairs (i.e. even numbers per nucleus), as if they had originated from a single DSB and then distantly separated. Taking a closer look, each partner of the foci pairs can be identified because each foci had a distinct size (Fig. 3B and reference photos provided in the supplemental data) [10]. In this case, the DSBs should have originated from the inside of the chromosome and not from the very end. Data in reports from Hewitt [36] and Fumagalli [35] indicates that, at most, 50% of unrepaired DSBs are located in telomeres; therefore, it is reasonable to assume that the causative mechanisms of unrepairable DSBs can be attributed to both their locations and chemical structures [63]. Collectively, while the fast phase of DSB repair constitutes rejoining of easy-to-repair 'clean DSBs', the slower phase might be composed of two distinct components, namely, telomere-silenced DSBs and unrepairable DSBs.

In yeast, several studies have indicated that persistent DNA lesions relocate to either the nuclear pore complex (NPC) or the nuclear envelope (NE) [64–66]. In such cases, specific membrane structures, including the components of nuclear pore proteins, appear to be crucial for the repair of persistent DSBs and eroded telomeres. In mammals, mutated forms of nuclear lamin A, referred to as progerin, induce deformation of the NE and impair certain processes of DSB repair. This process has been argued to contribute to the decrease in repair efficiency [67, 68] or to the enhanced production of unrepairable DSBs [69, 70]. In either case the expression of progerin has an adverse effect on DSB repair and induces premature senescence in cells. Likewise, NHDFs (normal cells) bearing radiation-induced unrepairable DSBs, which undergo premature senescence, show dysfunctional nuclear membrane structures [70]. These results indicate a possible link between unrepairable DSBs and premature senescence, which are both mediated by a dysfunctional nuclear membrane. The nuclear membrane and its periphery has been shown to associate with heterochromatin, where 'gene deserts' have also been shown to localize [71, 72]. The DSB repair of such regions is slow and dependent on both ATM and Artemis [51]. Overall, these data suggest that the final destination of unrepairable DSBs is the nuclear membrane. However, the nuclear periphery is also a place where telomeres locate [73] and is where telomere silencing occurs. Therefore, it is reasonable to speculate that both types of unrepairable DSBs, which primarily originated in either the telomere or heterochromatin, eventually colocalize in the nuclear membrane. New technologies [63, 74] and biomarkers will be necessary to distinguish radiation-induced unrepairable DSBs from those found at telomere ends. A further application of unrepaired DSBs measurement would be its use as an early indicator for radiation risk.

New methods that artificially introduce unrepairable DSBs at specific chromatin sites have been developed. White *et al.* [75] delivered Sac I restriction enzyme to mouse liver using adenovirus. They observed a liver-specific pathology of aging and inflammation. However, lipofuscin accumulation was not observed. Kim *et al.* [76] generated a conditional I-PpoI restriction enzyme expression system in mouse. In this system, 19 persistent DSBs could be formed in each cell, and the mice appeared to exhibit premature aging phenotypes. However, the introduction of such unrepairable DSBs could not fully explain all of the normal aging phenomena. The application of recent gene editing technologies will enable the introduction of unrepairable DSBs at specific sites in the chromosome. Such systems will help us to understand the risks of unrepairable DSBs in specific cells and tissues in living organisms.

CONCLUSION: A VIEW OF RADIATION BIOLOGY USING A NEW BIOMARKER

Although considerable efforts have been made to analyze the repair of repairable damage, studies that measure and elucidate the biology of unrepairable DSBs have not come to the forefront until recently. To better measure unrepairable DSBs, especially *in vivo*, we need definitive criteria for distinguishing unrepairable DSBs from transient and repairable DSBs. These criteria may include characterizing the precise sequences and structures where these breaks originated or new biomarkers that can specifically detect and measure these lesions. Such new technologies may enable the further application of radiation-induced unrepairable DSB quantification, which could lead to a better understanding of the risks of radiation to the processes of organismal development, growth, and aging.

SUPPLEMENTARY DATA

Supplementary data are available at the *Journal of Radiation Research* online.

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CONFLICT OF INTEREST

The author has no conflict of interest to disclose.

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