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The Yield, Processing, and Biological Consequences of Clustered DNA Damage Induced by Ionizing Radiation[#]

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Clustered DNA damage/Yield/Processing/Biological consequences/Non-DSB type/Ionizing radiation.

After living cells are exposed to ionizing radiation, a variety of chemical modifications of DNA are induced either directly by ionization of DNA or indirectly through interactions with water-derived radicals. The DNA lesions include single strand breaks (SSB), base lesions, sugar damage, and apurinic/apyrimidinic sites (AP sites). Clustered DNA damage, which is defined as two or more of such lesions within one to two helical turns of DNA induced by a single radiation track, is considered to be a unique feature of ionizing radiation. A double strand break (DSB) is a type of clustered DNA damage, in which single strand breaks are formed on opposite strands in close proximity. Formation and repair of DSBs have been studied in great detail over the years as they have been linked to important biological endpoints, such as cell death, loss of genetic material, chromosome aberration. Although non-DSB clustered DNA damage has received less attention, there is growing evidence of its biological significance. This review focuses on the current understanding of (1) the yield of non-DSB clustered damage induced by ionizing radiation (2) the processing, and (3) biological consequences of non-DSB clustered DNA damage.

INTRODUCTION

The high ionization density produced by radiation has long been related to the biological consequences. Howard-Flanders, a pioneer in the field of radiation biology, introduced the concept fifty years ago that a "lethal hit" to the cell can be defined in terms of the number of ionization events within a given target in the scale of several to several tens of nanometers. He argued that this "lethal hit" accounts for the linear energy transfer (LET) effects of radiation.[1)] Similar proposals were made in the following years,[2,3)] but they all failed to point out that the actual target of ionization is the DNA. The relevance and significance of the spatial distribution of DNA damage to its biological processing and consequences, such as repair, lethality, and mutation induction, were originally recognized from the biophysical considerations of Goodhead in the 1980s and 90s.[4–6)] He proposed that 1) spatial clustering of DNA damage is caused by dense ionization by ionizing radiation and 2) clustered DNA damage would be difficult to repair and would become a significant impediment to survival for the cell. Ward also concluded from radiation chemical point of view that the spatial distributions of the lesions are the key to describing the biological effectiveness of ionizing radiation. He demonstrated that the biological effectiveness of ionizing radiation is much higher than that of DNA oxidizing agents (such as H_2O_2), when identical amounts of damage are induced in cells.[7–9)]

Generally, clustered DNA damage is defined as two or more lesions formed within 1–2 helical turns of DNA by a single radiation track. A double strand break (DSB) is classified as clustered damage. DSBs are one of the most extensively studied types of cluster, probably because 1) they cause a change of the molecular weight of the irradiated DNA, which makes them easy to detect, and 2) a great deal of experimental evidence has accumulated over the years pointing towards a close correlation between unrepaired DSB and induction of chromosome aberrations, lethality etc.[10,11)]

Until recently, the occurrence and the processing of non-DSB clustered damage, which is composed of base lesions, AP sites, and strand breaks (that do not generate a DSB) were not clearly demonstrated in cells. Studies in the mid-70s and early 80s revealed an increase of the amount of DSBs during incubation after irradiation.[12,13)] This phenomenon was interpreted as a result of the action of some endonucleases against radiation-induced DNA damage. The

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increase of DSB post irradiation is now considered to be, at least in part, the outcome of the processing of ionizing radiation-induced non-DSB clustered damage. Further, the results of a number of studies have also led to the view that repair of radiation-induced non-DSB clustered damage is compromised. These studies demonstrated that in both cell extracts and cells, the rejoining of SSBs becomes less efficient as the LET increases.[14–17] However, until recently there has been little direct evidence that non-DSB clusters are induced by radiation or that they are relevant to the biological effectiveness of radiation. Significant progress has been made in recent years addressing these points. In the present review, we briefly summarize the recent progress on: 1) the yield of non-DSB clustered damage induced by ionizing radiation, 2) the processing of non-DSB clustered damage by repair enzymes, and 3) the biological consequences of non-DSB clustered damage.

As this review focuses on non-DSB clustered damage, we will simply refer to it as clustered damage hereafter. It is important to note that the types of lesions induced by radiation overlaps substantially with the types of lesions associated with endogenous oxidative damage, such as base lesions, AP sites, and strand breaks. Most, if not all, individual lesions, whether or not they are clustered or isolated, are considered to be recognized and processed by the base excision repair (BER) machinery, as depicted in Fig. 1.

The yield of clustered DNA damage

Clustered damage has long remained experimentally undetected, except for tandem lesions on the same DNA strand.[18–21] This was largely due to the lack of a simple but sensitive method to detect base lesions and AP sites that are spatially located close to each other.[22] The use of DNA glycosylases, such as Formamidopyrimidine glycosylase (Fpg) and Endonuclease III (Nth), whose major substrates are oxidized purines and oxidized pyrimidines, respectively, has made significant progress in the measurement of clustered damage. Because DNA glycosylases recognize base lesions and AP sites to create a SSB at the site of the damage through their AP lyase activity, bistranded clustered DNA damage, in which there is one or more base lesions or AP

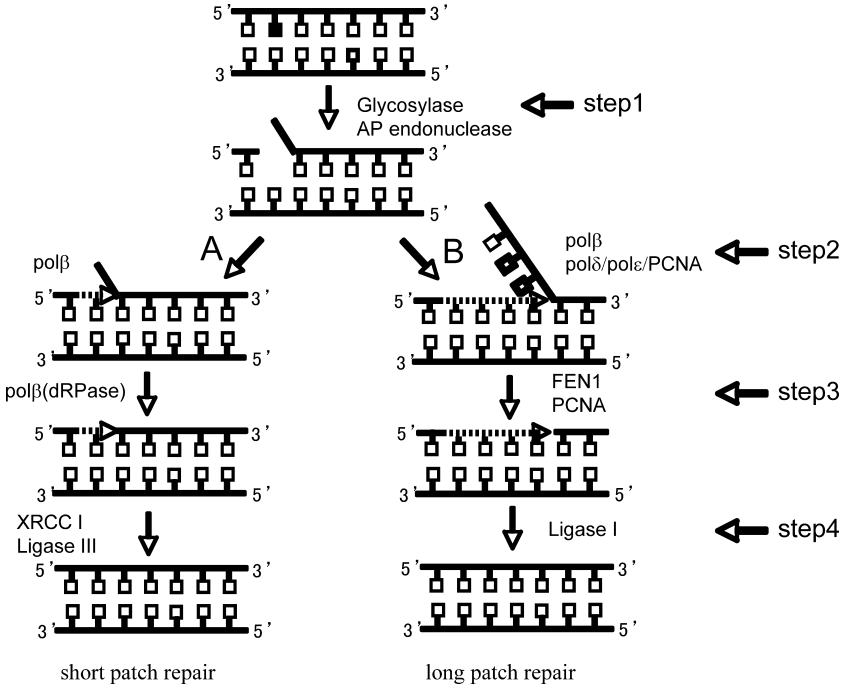

Fig. 1. Enzymatic steps of BER. In BER, glycosylases initially recognize the damaged base (black square) and excise the N-glycosidic bond to generate an AP site. A range of base lesions is recognized by several DNA glycosylases, which show broad specificities. The incision of DNA backbone at the AP site is incised by the accompanying AP lyase activity of the glycosylase or by the AP endonucleases (step 1) to generate a SSB. Blocks at the 3' end of SSB generated by the AP lyase activity are also removed by AP endonucleases. One or more nucleotides are inserted into the SSB by a DNA polymerase (step 2) followed by strand displacement (step3), and then the break is sealed by ligase (step 4). Depending on the number of nucleotide(s) inserted, BER proceeds through either of two subpathways, short patch repair or long patch repair.

sites on each strand, can be detected as a DSB after enzymatic treatment. Similar approaches to detect the clustering of AP sites have been made with AP endonuclease (Nfo) or polyamines (putrescine),[23] both of which generate a SSB at the site of the AP sites. Nfo and putrescine can cleave the DNA backbone not only at normal AP sites but also at oxidized AP sites that are generated by radiation. These techniques, in principle, transform non-DSB clustered damage into a DSB, which may then be used as a probe for clustered DNA lesions.

Computer simulations of radiation tracks can calculate the yield and the spatial distribution of DNA lesions. In addition to the experimental assay, these simulations have played a particularly important role in understanding the nature of ionizing radiation-induced clustered DNA damage and its dependence on LET. For instance, Nikjoo *et al*. showed that, in the case of proton-irradiation (LET = 9.2 keV/μm), 45% of the SSBs induced are accompanied by additional base lesions in close proximity *in vivo*, whereas the value increases to 78% after exposure to α-particles (LET = 129 keV/μm).[24] Other studies have also found a correlation between the complexity of strand breaks and LET.[25,26]

Several studies have estimated the yield of radiation-induced clustered DNA damage *in vitro* with phage or plasmid DNA, as well as *in vivo* with genomic DNA in mammalian cells and in animals, after irradiation with various kinds of radiation. Both the amount of water-derived radicals produced around the DNA and the LET of the radiation not only contribute to the yield of clustered damage but also to the ratio of clustered to isolated lesions.[27–33] In cells or under cell-mimetic conditions, where scavenging capacity of the DNA solution is equivalent to that of the cells, the amount of each type of clustered damage revealed by the treatments of different repair enzymes is comparable to or slightly less than that of DSB (Table 1).

There is no clear dependence on LET for DSB in cells, whereas simulation studies and experimental results of plasmid assays under cell-mimetic conditions indicate an increase in the yield of DSB with increasing LET up to 100–200 keV/μm.[34,35] The reason for this discrepancy needs to be clarified, but experimental biases might have affected the yields of DSB. In pulsed field gel electrophoresis (PFGE), DNA fragments less than 10 kbp are normally excluded, and thus the DSB yields in genomic DNA are most likely underestimated to variable extents.[36,37] An additional factor that may need to be taken into account is the fraction of DSBs that originated from heat-labile sites. This fraction is significant especially when DSBs are measured by PFGE, because genomic DNA is usually exposed to elevated temperatures during the lysis of cells.[38–40] Since bistranded clustered damage sites are detected as DSBs, comparisons between the yield of bistranded clustered damage in cells and that *in vitro* need to be made with caution. Additionally and perhaps more importantly, the above experimental assay to reveal non-DSB clustered damage carries an intrinsic limitation, which is that the capacity of repair enzymes to cleave at a lesion within a cluster is reduced in most cases (see next section for details). For example, the frequency of abasic clusters revealed by Nfo is much lower than that revealed by putrescine.[23] This limitation greatly hampers accurate measurements of clustered lesions, especially at higher LETs, as the complexity of the cluster increases so that the enzyme efficiencies may decrease.

Although the accurate yield of non-DSB clustered DNA damage after irradiation, especially with high-LET radiations, still remains to be determined experimentally, biophysical simulations can provide an initial estimate of the LET dependence of the amount of clustered DNA damage. Nikjoo *et al*. calculated that base lesions are invariably 2-2.4 fold more abundant than strand breaks after irradiation with protons and helium ion particles with LETs ranging from 9.2

Table 1. Yields of radiation-induced clustered DNA damage in cells or under cell mimetic conditions

radiation	cell/DNA	method (analysis)	DSB[a]	endoIV[a,b]	Fpg[a,b]	endoIII[a,b]	ref
γ	Chinese hamasterV79	PFGE (FAR)	1.1		1.3	0.91	41
γ	human fibroblastsHF19	PFGE (FAR)	1.9		0.67		41
		PFGE (hybridization)	0.85			0.64	41
γ	human monocytes 28SC	PFGE (number average length analysis)	1.8	1.5	1.8	1.6	37
Fe (LET = 148 keV/μm)	human monocytes 28SC	PFGE (number average length analysis)	1.7	0.85	1.3	1.1	37
γ	plasmid pUC18	GE	1.6		1.3	1.3	41
γ	plasmid pMSG-CAT	GE	1.1			4.6	85
α (LET = 110 keV/μm)	plasmid pMSG-CAT	GE	2.3			0.77	85

[a]: yields are shown as $X10^{-11}$/Gy/Da.

[b]: yield of clustered DNA damage revealed after treatment with respective repair enzymes.

to 168 keV/μm under cell-mimetic scavenger conditions.[24] If the yield of DSB increases with increasing LET in this range in cells, one may further expect, from the stable ratio of the amount of base damage to strand breaks, that the yield of non-DSB clustered DNA damage would decrease relative to the DSB yield. The validity of this prediction must await experimental evidence. Recent experimental studies have estimated that the yield of clustered damage after low LET-irradiation in cells is at least 3–4 times larger than that of DSB.[41,42]

In vitro processing of clustered DNA damage

Although a considerable amount of clustered DNA damage appears to be induced in cells, how and to what extent clustered DNA damage is processed has long remained unknown, probably because 1) the yield and the nature of radiation induced-clustered DNA damage were difficult to measure experimentally (as discussed above) and 2) damage sites are induced randomly by ionizing radiation. Our current understanding of the processing of clustered DNA damage is largely obtained from *in vitro* studies that analyze the capacity of synthetic model clusters to be repaired, using either purified enzymes or cell extracts. To overcome the random nature of radiation-induced changes, many research groups have used synthetic clusters, in which the type, number, and relative position of the lesions are specified, for examining how the clusters are processed. Various types of synthetic base lesions, such as 7,8-dihydro-8-oxoguanine (8-oxoG), thymine glycol (Tg), dihydrothymine (DHT), dihydrouracil (DHU), 5-hydroxyuracil (5-OHU), as well as AP sites, have been subjected to analysis. The majority of these studies examined synthetic clusters comprised of two lesions and have generally focused on determination of the efficiencies of either the individual steps of BER/single strand break repair (SSBR) or the BER/SSBR pathway as a whole.

The initial step of BER processing, which is the excision of base lesions or incision of AP sites within a bistranded or tandem cluster by various glycosylases (Fpg, Nth, OGG1 etc.), AP endonucleases (exonuclease III, APE1, etc.), and by cell extracts (Fig. 1, step1), has been extensively studied (see reviews).[43–45] It may be worth mentioning here that 1) yeast and human OGG1 are functional homologues of bacterial Fpg, 2) yeast Ntg1, Ntg2, and human NTH1 are functional homologues of Nth, 3) human NEIL1 and NEIL2 are functional homologues of Nei, and 4) human APE1 is a functional homologue of exonuclease III. A general view obtained from studies with bistranded and tandem damage sites is that

1) the excision of a base lesion is not strongly retarded by a lesion on the opposite strand unless the lesions are located within 1bp;
2) the excision of a base lesion is strongly retarded by an AP site or a SSB on the opposite strand when lesions are within 3–5 bp separations;
3) the excision of an AP site is retarded only when another AP or SSB resides 5' to each other within 3–5 bp separations on the opposite strand, and not by a base lesion;
4) the excision of an AP site is strongly retarded by a base lesion on the same strand when lesions are confined within 3–5 bp.

These results imply that once a base lesion is cleaved and an AP site/SSB is formed, the processing of other base lesions located within 3–5 bp will be compromised. In contrast, a bistranded cluster that has lesions > 5 bp apart could be readily converted to a lethal DSB through processing by BER/SSBR (Fig. 2, pathway B). It is important to note that the effect of spacing and polarity of lesions on the incision activity has often been correlated with the biochemical and structural properties of an enzyme. For instance, Wilson *et al*. demonstrated that APE1 requires a double-helical structure for at least 4 bp on the 5' of the AP site but only 3 bp on the 3' side, consistent with the observation that clusters with AP sites positioned 5' to each other are poor substrates of APE1.[46] Structural and chemical studies have helped to elucidate interactions of enzymes with DNA,[47–49] and will also help to explain the efficiency of cleavage at specific configurations of lesions. Although the results of clusters comprised of two lesions have become the basis for understanding the initial processing of clustered DNA damage, further work is required to uncover what the outcome will be with more complex clusters. Interestingly, in this context, Paap *et al*. have recently reported that the formation of DSBs would be greatly reduced when any two of the three AP sites are located within 3 bp to each other in the 5' direction.[50] Likewise, Eot-Houllier *et al*. have demonstrated that the sequential excision of base damage prevents the formation of a DSB in complex clustered damage comprised of a gap

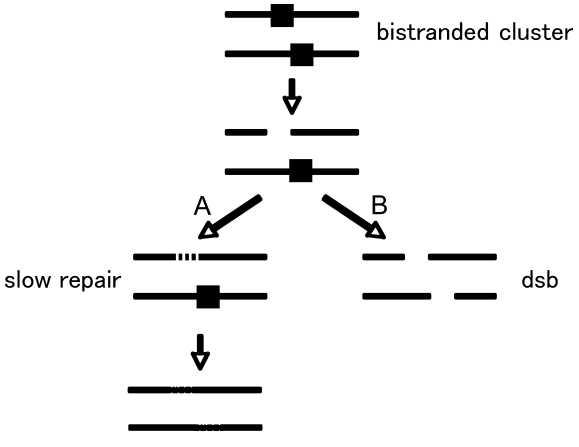

Fig. 2. The processing of a bistranded cluster. In pathway A, a damaged site is processed and repaired sequentially by the BER pathway to avoid the formation of a DSB. In pathway B, damaged sites are processed simultaneously prior to completion of repair resulting in the formation of a DSB.

and 3–4 base lesions.[51,52] In addition to the retardation of enzymatic activities of glycosylases and AP endonucleases during the initial step of BER, the efficiencies of the latter steps for either short or long patch repair of BER/SSBR (Fig. 1, steps 2–4) are often reduced for various types or configurations of clustered damage. For instance, both nucleotide insertion (Fig. 1, step 2) by polβ and ligation (Fig. 1, step 4) by ligase IIIα are compromised in the short patch repair pathway after incision of an AP site opposed to Tg.[53] Placing 8-oxoG or 5-OHU on the same strand proximal to or at the 3' end has been shown to also retard the polymerization and ligation steps at a SSB.[54–58]

Interestingly, several *in vitro* studies have shown that the overall efficiency of repair could be quite different between clusters, even with those that would be potentially processed into the same repair intermediate. One example was observed with tandem lesions containing 8-oxoG. Cunniffe *et al*. demonstrated that, when an AP site is placed upstream (5') of an 8-oxoG in tandem, the repair of the AP site was strongly impaired up to 4-fold.[58] On the contrary, no apparent retardation of repair of an upstream (5') 8-oxoG was found in a configuration with two 8-oxoGs placed in tandem.[54] Even though the same repair intermediate, a SSB with 8-oxoG located proximal to the 5' end, is probably formed in both cases, the results demonstrate that the reparability of these two upstream (5') lesions are quite different. This difference suggests that the interplay between repair proteins plays a crucial role during the processing of clusters. Different reparability of clusters with similar configurations is also found in the case of bistranded clusters. Lomax *et al*. showed that, with constructs having an AP site opposite to an 8-oxoG, the level of ligation at the AP site was reduced up to 2-fold when compared with that for a single AP site. They further found that the repair efficiency of a SSB was ~ 4 times less for that of an AP site when the lesion was placed in a bistranded cluster containing 8-oxoG.[59,60] The more efficient repair of the AP site within a cluster was probably due to 1) the greater efficiency of short patch repair for an AP site than for a SSB and 2) the strong retardation of the ligation step during the long patch repair for a SSB.[61] This observation, in which an AP site (in contrast to a SSB) opposed to an 8-oxoG was efficiently processed by short patch repair, was consistent with the results of earlier studies.[51,62] It remains to be resolved whether processing steps other than ligation are impaired in long patch repair. With a reduced AP site positioned one nucleotide 3' opposed to an 8-oxoG, Budworth concluded that the retardation of long patch repair after incision of the AP site was due to 1) ineffective strand displacement by polymerases, polβ or polδ and 2) ineffective cleavage of the flap by FEN1.[62] In contrast, using similar clusters containing an AP site and 8-oxoG, Morgues *et al*. observed no retardation in cleaving the flap by FEN1 but did observe retardation in the final ligation step involving ligase I.[61]

In vitro studies based on the efficiency of BER in cell extracts suggest that in the processing of base lesions within clustered DNA damage, there is a hierarchy of repair of lesions within the cluster. For instance, Lomax *et al*. found that 8-oxoG is hardly incised during the repair of an AP site or SSB on the opposite strand (Fig. 2, pathway A).[59,60] Similarly, placing five different types of lesions within close proximity to each other, Eot-Houllier *et al*. clearly demonstrated that a preferential order for incision of the lesions is present and that the repair proceeds sequentially in human fibroblast cell extracts.[51,52] The existence of a hierarchy also points toward the biological importance of the complex interplay between various repair proteins to avoid the generation of a DSB that could be deleterious to cells. Interestingly, no such clear order for repair was seen with clusters comprised of two AP sites. When bistranded AP sites were separated by less than 5 bp, 85% of the clusters were converted to DSBs in CHO cell extracts (Fig. 2, pathway B).[59] Only when the two bistranded AP sites are within 1 bp located 5' to each other is the conversion to a DSB retarded. These studies strongly suggest that the type of the lesions within a cluster determines the outcome.

An alternative approach to understand the processing of clustered damage is to specify the repair enzyme dealing with a cluster. Venkhataraman *et al*. found that removal of a DHU at the 3' end of a SSB can be carried out with DNA glycosylases such as human NTH1 and *E. coli* Nei, but not with *E. coli* Nth, Yeast Ntg1p or Ntg2p. On the other hand, it was revealed that Yeast Ntg2p, but not *E. coli* Nth, Yeast Ntg1p, human NTH1 or *E. coli* Nei, could cleave DHU located at the 5' end.[63] Similarly, in human cells, an AP endonuclease (APE1) or NEIL1 was shown to be the major activity to remove 8-oxoG, DHU or 5-OHU at or near the 3' end of a SSB.[55,64,65] Interestingly, the major enzyme in human cells involved in the removal of 5-OHU when positioned as the second nucleotide from the 3'-end of a SSB is polδ, but when 5-OHU is positioned as the third or fourth nucleotide from the 3'-end, it is DNA glycosylase hNEIL1.[56,57,65] It should be noted that hNTH1 cannot remove 5-OHU located as the second, third, and fourth nucleotide from the 3'- end. Recently, the XRCC1 protein, which is proposed to act as a scaffold protein that coordinates the enzymatic processing in BER or SSBR, was shown to be required for the repair for an AP site opposite 8-oxoG, but not for the less efficient repair of SSB in a SSB/8-oxoG cluster.[61]

The above information on the processing of clustered DNA damage suggests that the presence of one or more vicinal lesions affects the rate or fidelity of DNA repair as well as the types of enzymes involved. It is worth noting that in most, if not all, cases, the repair of clustered lesions is retarded compared with the repair of isolated lesions. The less effective repair of lesions in a cluster should not be simply considered as deleterious or harmful to cells, as it may

often protect against the formation of lethal DSBs. A major consequence of retarded processing of clustered DNA damage is the extended lifetime of lesions within the cluster so that the cluster may be present at replication. Other aspects of the processing of clusters, such as the coordination of repair and the formation of DSB as a repair intermediate, would also strongly affect the biological consequences of the clusters. The current understanding of *in vivo* processing of clustered damage and its biological consequences will be further discussed below.

In vivo processing and biological consequences of clustered DNA damage

Our understandings of the *in vivo* processing of cluster DNA damage induced by ionizing radiation and of the subsequent biological consequences have greatly increased in recent years. The processing of radiation-induced clustered DNA damage in cells has been inferred through the enzymatic formation of DSBs from clustered DNA damage. Although the generation of additional DSBs after exposing cells to ionizing radiation has been known for over 30 years,[13,66] Blaisdell and Wallace were the first to demonstrate that, using *E. coli* cells, the level of additional DSBs generated post-irradiation was dependent on the amount of glycosylase (Fpg) and was related to lethality.[67] Yang *et al*. further demonstrated that, in human B lymphoblastoid cells, the induction of DSBs, mutations, and lethality all strongly correlated with the expression levels of glycosylases (hOGG1 and hNTH1) after exposure to 3 Gy of γ-rays.[68,69] With NHEJ-deficient CHO *xrs-5* cells, Gulston *et al*. showed that, after irradiation of γ-rays, only a fraction (~10%) of clustered DNA damage is actually converted to DSBs.[40] However, such additional DSBs are not always observed. For instance, DSB repair-proficient Chinese hamster V79 cells and human hematopoietic cells did not generate additional DSBs during post-irradiation with γ-irradiation.[40,70] Detection of the additional DSBs at high doses (~500 Gy) in previous studies[13,66] implies the presence of an abortive enzymatic repair of clusters *in vivo*. The observations that not all of the clustered DNA damage sites are converted to DSBs at lower doses in NHEJ-deficient cells and the finding that the additional DSBs are not formed in NHEJ-proficient cells suggest that cells have mechanisms to avoid DSB formation or to quickly rejoin *de novo* DSBs through NHEJ and alternative pathways. It is important to note that enzymatic processing of clustered lesions has not been observed after irradiation with high-LET radiations. In the case of wild-type Chinese hamster V79 cells, the amount of DSBs remained essentially constant after α-irradiation.[40] Further, Chang *et al*. did not observe any enhancement of cell killing after exposing an Fpg-overexpressing *E. coli* strain to α-rays.[71] These results may reflect the greater complexity of clustered damage sites generated by high-LET radiation, in which the lesions are unable to be processed by repair enzymes.

The fact that the amount of clustered damage eventually decreases during post-irradiation incubation provides further evidence for the *in vivo* processing of cluster damage.[37,40,70] However, the reliability of this conclusion is unclear, because the enzymatic processing of clustered damage is not the only way to reduce the level of clustered lesions. The amount of clusters could also be reduced by elimination of cells by cell death, and/or a "diluting effect" through replication.[37,70] Indeed, in human hematopoietic cells and monocytes, the amount of bistranded AP sites seems to be reduced mainly via "dilution" through replication. Further studies are needed to clarify mechanisms of *in vivo* processing of radiation-induced cluster damage through activities other than the formation of de novo DSBs and the decrease of the amount of clusters.

The biological relevance of clustered damage has been difficult to demonstrate, due to its random nature of radiation-induced lesions in terms of the types, numbers, and relative positions. Therefore, whether a clustered DNA damage site is indeed relevant to biological endpoints has mostly been investigated with model synthetic lesions. The mutagenic potential of an 8-oxoG with another base lesion or an AP site within close proximity on the opposite strand is enhanced in *E. coli* cells.[72–75] In these studies, the majority of the mutations found are G-to-T transversions at 8-oxoG sites, with few deletions. In addition, the transformation efficiency of bacteria with plasmids carrying these clusters is comparable to that of undamaged DNA, which indicates that most of these clusters are not processed into DSBs during repair. The *in vivo* results of these model bistranded damage sites are in good agreement with those of *in vitro* studies, and indicate that 1) the repair of an 8-oxoG is retarded, so that some of the clusters remain partly unrepaired by the time of replication, 2) lesions are repaired sequentially and the formation of DSBs is minimized, and 3) there appears to be a preferential order for excising different lesions. On the contrary, the mutagenic potential of an 8-oxoG placed in tandem with an AP site remains similar to that of a single 8-oxoG in wild-type cells of *E. coli*, and even decreases in *fpgmutY* cells.[58] This implies that the position of the base damage and the existence of an undamaged strand greatly influence the mutagenic consequences of a cluster. With bistranded uracils, which are considered to be quickly converted to bistranded AP sites *in vivo*, the induction of DSBs through the processing of clustered DNA damage in *E. coli* has been inferred from the formation of deletions.[76] In addition, a reduction of transformation efficiency, which implies the formation of DSBs, was also observed when the two uracils were separated by less than 7 bp.[77] Puzzlingly, the reduced transformation efficiency was still found in the absence of enzymatic activities for incision of an AP site, such as AP endonucleases (exoIII, endoIV, and endoV), AP lyases (endoIII, Fpg, endoVIII), and NER (UvrA).[78] It was

recently shown in mouse cells that two opposed tetrahydrofurans can be cleaved into a DSB by AP endonuclease(s) and a fraction of the lesions can be inaccurately repaired by NHEJ resulting in deletions.[79] A tetrahydrofuran is a stable AP site analog. This formation of DSB in mammalian cells is consistent with the results from *in vitro* processing of bistranded AP sites in cell extracts.

Studies of *in vivo* processing of clustered damage and its relevance to biological consequences have confirmed the significance of the results revealed *in vitro*, such as the retardation and hierarchy, and formation of deleterious DSB during repair. It is important to consider the possibility that additional factors which modify the induction or processing of clustered damage have a role *in vivo*. For instance, subpathway(s) or modulating factors of BER might play a key role in the processing the clusters. It has been proposed that HU$\alpha\beta$ in bacteria and Ku and possibly poly(ADP-ribose) polymerase-1 (PARP-1) in mammalian cells are involved in preventing the formation of DSBs.[80,81]

SUMMARY AND PERSPECTIVES

Our understanding of the biological significance of non-DSB clustered damage has greatly progressed in recent years. It is now widely accepted that, in cells, 3–4 times more bistranded non-DSB clusters are produced than DSBs with low-LET radiation, although the yields of clustered lesions vary depending on the experimental assay used to measure DSBs. Detailed *in vitro* investigations have revealed the importance of the processing of clustered damage. The repair of the lesions within the cluster is impaired to various extents and at various steps of BER/SSBR and that the types and configurations of lesions within a cluster strongly affect the outcome (eg. DSB formation). Later studies indicated that the interplay between repair proteins affect the outcome of the processing of the clustered damage, and thus would also strongly influence the biological consequences. Further, *in vivo* experiments have confirmed that clustered damage has greater biological effects, such as lethality and mutagenicity, than isolated single lesions.

Despite these extensive efforts, the exact nature of the radiation-induced clustered damage has yet to be determined experimentally and it is still unclear how much clustered DNA damage is caused by a high-LET radiation. The processing of the cluster and its outcome also need further investigation. For instance, further studies are needed to predict what the outcome would be after the processing of clusters comprised of an untested configuration of lesions and at complexities predicted for high LET radiations. In addition to the processing of clusters, the effect of unrepaired clusters on the progression and fidelity of replication needs to be taken into account to explain how clustered DNA damage is related to the frequency and the nature of chromosomal aberrations and mutations induced by radiation (especially of high LET).[82–84] Further and more detailed classification on the type of the cluster according to how and to what extent the damage is processed under various conditions would help to reveal the processing mechanisms as well as the biological significance of clustered DNA damage.

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