Role of isolated and clustered DNA damage and the post-irradiating repair process in the effects of heavy ion beam irradiation

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ABSTRACT

Clustered DNA damage is a specific type of DNA damage induced by ionizing radiation. Any type of ionizing radiation traverses the target DNA molecule as a beam, inducing damage along its track. Our previous study showed that clustered DNA damage yields decreased with increased linear energy transfer (LET), leading us to investigate the importance of clustered DNA damage in the biological effects of heavy ion beam irradiation. In this study, we analyzed the yield of clustered base damage (comprising multiple base lesions) in cultured cells irradiated with various heavy ion beams, and investigated isolated base damage and the repair process in post-irradiation cultured cells. Chinese hamster ovary (CHO) cells were irradiated by carbon, silicon, argon and iron ion beams with LETs of 13, 55, 90 and 200 keV µm⁻¹, respectively. Agarose gel electrophoresis of the cells with enzymatic treatments indicated that clustered base damage yields decreased as the LET increased. The aldehyde reactive probe procedure showed that isolated base damage yields in the irradiated cells followed the same pattern. To analyze the cellular base damage process, clustered DNA damage repair was investigated using DNA repair mutant cells. DNA double-strand breaks accumulated in CHO mutant cells lacking Xrcc1 after irradiation, and the cell viability decreased. On the other hand, mouse embryonic fibroblast (Mef) cells lacking both Nth1 and Ogg1 became more resistant than the wild type Mef. Thus, clustered base damage seems to be involved in the expression of heavy ion beam biological effects via the repair process.

KEYWORDS: clustered DNA damage, oxidative base lesion, heavy ion beam, high-LET radiation, relative biological effectiveness (RBE)

INTRODUCTION

Over the last century, heavy ion beams have been used for cancer therapy due to their effective cell killing ability, and we have commenced use of an environment filled with space radiations. There are increased risks associated with heavy ion beam irradiation because heavy ion beams have more critical biological consequences for living organisms than the more commonly used gamma- and X-rays [1]. However, we have little information regarding the molecular mechanisms underlying the biological effects of heavy ion beams. Ionizing irradiations cause various kinds of damage to biomolecules (such as proteins, membrane lipids, and nucleic acids). These damages are deeply involved in the radiobiological effect. Of the biomolecules, DNA seems to be the main target of heavy ion beam irradiation. Because DNA harbors the genetic information in most living organisms, elucidation of the difference between DNA damage induced by heavy ion beams and gamma (X)-rays may explain why heavy ion beams have more critical biological consequences.

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Ionizing radiations traverse the target molecule as beams. Hence, the ionizing radiation damages the target molecule along its track. When the target molecule is DNA, such damage aggregation is termed as ‘clustered DNA damage’ or ‘multiply damaged sites’, and is known as a specific form of DNA damage induced by ionizing radiations [2, 3]. Clustered DNA damage is presently defined as a damage complex consisting of two or more lesions gathered in two helical turns (a 20-base pair-length) of double-strand DNA [4]. However, the nature of the component lesions forming clustered DNA damage remains undefined. In fact, all kinds of radiation DNA damage, including oxidative pyrimidine and purine lesions, abasic (apurinic/apyrimidinic: AP) sites, and strand breaks, may constitute clustered DNA damage [2, 3]. A DNA double-strand break (DSB) is thought to consist of two adjacent single-strand breaks (SSB) on opposite DNA strands and to represent one kind of clustered DNA damage. For a long time before the conception of clustered DNA damage, a DSB was considered as typical DNA radiation damage. Thus, we have accumulated a large amount of knowledge pertaining to ionizing radiation-induced DSBs. Then we recognized that a DSB is a well-known type of malignant DNA damage, which can inhibit replication and which possesses a mutagenic ability [5]. In contrast, clustered DNA damage containing base lesions (clustered base damage) remains undefined, and may have more complex biological outcomes. Until now, clustered base damage has been less studied, although it was reported that clustered base damage yield is considered to be more than double that of DSBs [2].

In response to this situation for clustered DNA damage, especially clustered base damage, we designed this study to elucidate the mechanisms underlying the biological effects of heavy ion beam radiation on clustered base damage. First, we determined clustered base damage yields in irradiated cells, and then the involvement of the repair process. Previously, we estimated clustered DNA damage yields in pDEL19 plasmid (a super-coiled circular DNA) and lambda phage DNA (a linear DNA) irradiated with gamma-rays, and carbon and ion beams in test tubes [6]. In that study, clustered DNA damage yields decreased with the increase in linear energy transfer (LET), which is in agreement with other studies [7]. Thus, the quantity of clustered DNA damage is not the only explanation for the severity of the biological effects induced by heavy ion beams. In the present study, we analyzed clustered base damage yields in cells irradiated by heavy ion beams. This study indicates that clustered DNA damage yields, including clustered base damage, also decreased with increasing LET in the irradiated cells. Thus, not only the quantity but also the quality and/or its secondary products seem to be important for the expression of the biological effects, explaining the severity of the biological effects induced by heavy ion beams.

Among the potential factors, the repair process of clustered base damage attracted our attention. In fact, a certain type of clustered base damage can be transformed to DSBs after base excision repair (BER) [8]. During the BER process, for an isolated base lesion, an appropriate DNA glycosylase first recognizes the base lesion on the DNA and degrades the N-glycosidic bond between the base and the sugar [9]. Subsequently, an AP endonuclease or a DNA glycosylase cut the DNA strand at the AP site generated by the first step, and a DNA strand break is produced. The strand break is known as an inevitable and unwelcome reactive intermediate during BER. If two or more base lesions are concentrated in a limited area on a DNA double helix, and at least a pair of these lesions is separated to the respective strands (bistranded clustered base damage), the simultaneous motion of BER activity to these lesions generates DSBs. Thus, clustered base damage is a latent DSB, and its malignant potential is induced via the repair process [6, 10–12].

In view of the above, we have three aims leading on from our previous study in this field. First, we focused clustered base damage in the cells irradiated by heavy ion beams. In the previous study, we investigated clustered DNA damage of the irradiated DNA molecules in a test tube [6]. The investigation of cellular clustered DNA damage is necessary in order to discuss the biological impact of heavy ion beams, but not the molecular impact in a test tube. Second, we used more types of radiation in this study than in the previous study in order to generalize our concept. We added silicon and argon ion beam radiation types in this study. Third, we examined the repair process in order to discuss the molecular mechanism of biological effect with heavy ion beams via clustered base damage. Hence, we irradiated wild type Chinese hamster ovary (CHO) cells with carbon, silicon, argon and ion beams in this study. To estimate clustered DNA damage (including clustered base damage), the irradiated cells were subjected to static field gel electrophoresis (SFGE) with treatments of the appropriate DNA glycosylases, corresponding to specific oxidative base lesions. We also measured the isolated base damage in the irradiated cells using the aldehyde reactive probe (ARP) method with appropriate DNA glycosylases, and we found a result like clustered base damage as mentioned above. The surviving ratio of irradiated cells decreased as the LET increased, which means that biological effect of heavy ion beams intensifies with the LET increase. Thus, damage yield decrease was inversely proportional to the irradiated cell’s ability to survive. It suggests that the quality of clustered base damage is just a factor or is not important for the biological effect of heavy ion beams. Therefore, we next investigated the repair process of clustered base damage in irradiated cells mutated for BER components. During the post-irradiation culture period, DSB accumulation was observed in CHO cells lacking Xrcc1, which is the gene coding for the scaffold protein involved in the BER-specific ligation step [13]. The cells were also more sensitive to ionizing radiation. To investigate the role of the DNA glycosylase step, mouse embryonic fibroblast (Mef) mutated for Nth1 and Ogg1 (which are the specific DNA glycosylases for oxidative pyrimidines and purines, respectively [14, 15]) was used, and it presented an increased resistance to ionizing radiation. These results indicate that, first, clustered base damage in irradiated cells can be processed by BER, and produce DSBs as an intermediate. Second, the intermediate DSB seems to increase the irradiated cell death. Taken together, our present results suggest that clustered base damage contributes to the biological effect of heavy ion beams, both directly and indirectly with its biological processes.

**MATERIALS AND METHODS**

**Materials**

Both CHO-AA8 wild type and the mutant EM9 lacking Xrcc1 cells [16, 17] were laboratory stocks. Mef cells mutated for Nth1 and Ogg1 were created in a group member’s laboratory (A.Y.). *Escherichia coli* endonuclease III (EndoIII), formamidopyrimidine DNA glycosylase (Fpg), and human 8-oxoguanine-DNA glycosylase (hOGG1) were prepared, as described previously [18, 19]. Pulse field gel certified agarose was purchased from Bio-Rad (Hercules, CA, USA) to prepare the SFGE gel. SeaPlaque GTG Agarose from Cambrex (East Rutherford, NJ, USA) was used for the SFGE agarose plug. ARP was from the University of California, San Francisco, CA, USA.
The chemicals used in this study were from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise stated.

Irradiation

CHO cells were cultured in minimum essential alpha medium (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) in a 5% (v/v) CO2 atmosphere at 37°C. Mef cells were cultured in Dulbecco’s modified Eagle medium + GlutaMAX (Gibco) with 10% (v/v) FBS.

For SFGE analysis of clustered base damages, the cells were seeded at a concentration of 1 × 10^4 cells per 25-cm² flask, and grown for 24 h prior to irradiation. The cultured cells were irradiated with carbon, silicon, argon and iron ion beams at the Heavy Ion Medical Accelerator in Chiba (HIMAC) of the National Institute of Radiobiological Sciences, Japan. The energies of carbon, silicon, argon and iron ion beams were 290, 490, 500 and 500 MeV/u, respectively. The LETs were 13, 55, 90 and 200 keV µm⁻¹, respectively. The dose rates were 4 Gy min⁻¹ for carbon, 7 Gy min⁻¹ for silicon and argon, and 15 Gy min⁻¹ for iron ions. The cells were kept at 4°C with a cooling apparatus during the irradiation. For the repair analysis, the irradiated cells were recovered after the post-irradiation culture, as described below. For ARP analysis of isolated base damages, the confluent cells grown in a 75-cm² flask were harvested by trypsinization with 0.05% Trypsin-EDTA (1 × ; Gibco) and centrifugation. The cell pellet was irradiated in a microtube. The irradiated cell pellet was promptly frozen on dry ice and stored at −80°C until the analysis. The colony-forming units of the irradiated surviving cells were estimated, as described previously [6].

We also exposed the cells to gamma-rays using a ¹³⁷Cs gamma-ray source (Atomic Energy of Canada Ltd, Chalk River, ON, Canada) at Saga University for a comparative experiment. The LET was 0.9 keV µm⁻¹ and the dose rate was 0.8 Gy min⁻¹ for the gamma-rays.

Analysis of clustered base damage by SFGE

Clustered base damage in irradiated cells was analyzed using the procedure reported previously with additional enzyme treatments, as mentioned below [6]. The irradiated cells were recovered with trypsinization, and embedded in 1% (w/v) agarose gel plugs (5 mm × 1 mm × 7 mm). The gel plugs were incubated in 2 ml of lysis solution (Trevigen, Gaithersburg, MD, USA) containing 0.5 mg ml⁻¹ of proteinase K at 50°C for 24 h, and then rinsed in 10 ml of TE buffer (pH 8.0) consisting of 10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl, 0.5% (w/v) bovine serum albumin (BSA). After these treatments, the plugs were incubated in the lysis solution containing 0.5 mg ml⁻¹ of proteinase K at 50°C for 2 h to inactivate the DNA-modifying enzymes. After washing twice in TE buffer at 25°C for 15 min, the plugs were inserted in 0.6% (w/v) agarose gel, and electrophoresed at 17 mA of constant current in TBE buffer (90 mM Tris, 90 mM boric acid, and 2 mM EDTA) for 20 h. The image of electrophoresed gel stained with 2.5 µg ml⁻¹ of ethidium bromide in TBE was captured on a digital photograph device STAGE-1000 (AMZ System Science, Osaka, Japan), and the fluorescent bands (corresponding to intact and damaged chromosomal DNA, indicating the generation of clustered DNA damage in the irradiated cells) were quantified by using Image J software [20].

Analysis of isolated base damage by ARP

ARP analysis for isolated base damage was performed as reported previously [21]. The chromosome DNA was isolated from the irradiated cells by the sodium iodide (NaI) method [22]. The DNA was dialyzed against TE buffer overnight at 4°C to remove residual NaI. For the detection of pyrimidine and purine damages, 15 µg of the treated DNA was incubated with EndoIII (1.5 µg) or hOGG1 (1.5 µg) in the DNA glycosylase reaction buffer (total volume: 90 µl) at 37°C for 1 h. After the enzymatic treatment, the DNA was recovered by ethanol precipitation. Next, 10 µg of DNA was incubated with 5 mM ARP in TE buffer (pH 7.5) at 37°C for 10 min. The ethanol precipitation was then repeated. Recovered ARP-labeled DNA was heated at 100°C for 5 min in 10 mM Tris-HCl and 0.2 mM EDTA (pH 7.5), and quickly cooled on ice. The DNA solution was mixed with an equivalent amount of 2 M ammonium acetate, and 1 µg of the ARP-labeled DNA was then blotted on a Biodyne Plus membrane (Pall, Pensacola, FL, USA) using a slot-blower with vacuum. The DNA was immobilized on a membrane by UV irradiation (12 mJ as the total fluence). The membrane containing fixed DNA was washed at 30°C for 1 h in Tris-NaCl-BSA buffer consisting of 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, 0.5% (w/v) casein, 0.25% (w/v) BSA, and 0.1% (w/v) Tween 20. The membrane was then soaked in 20 ml Tris-NaCl-BSA buffer containing 16 µl of the preformed ABC reagents (Vector Laboratories, Burlingame, CA, USA) for 45 min. After washing again with 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.26 M NaCl, and 0.1% (w/v) Tween 20, the membrane was incubated in ECL detection reagent (GE healthcare, Wauwatosa, WI, USA). The chemiluminescence was immediately measured on a luminescent image analyzer LAS-3000 (Fujiﬁlm, Tokyo, Japan) equipped with a CCD camera. The signal was integrated for 1 h and quantiﬁed by using the Multi-gauge software (Fujiﬁlm) provided with the analyzer.

RESULTS

In this study, we investigated whether our results in the test tube [6] were transposable to cells irradiated by heavy ion beams in order to elucidate the role of clustered DNA damages in the radiobiological effect of heavy ion beams. Cell sensitivity to ionizing radiations, including gamma-rays and heavy ion beams, was first assessed by a colony-forming assay. The survival of irradiated CHO-AAB cells decreased with the increase in LETs (Fig. 1). The D10 of the irradiated cell survivals were 5, 3.7, 2.4, 2.2 and 1.95 Gy for gamma-rays, carbon, silicon, argon and iron ion beams, respectively. LETs were 0.9 keV µm⁻¹, 13 keV µm⁻¹, 55 keV µm⁻¹, 90 keV µm⁻¹ and 200 keV µm⁻¹ for gamma-rays, carbon, silicon, argon and iron ion beams, respectively. These results indicate that the ionizing radiation...
LET is directly linked to the relative biological effect (RBE) of the irradiation.

We then analyzed clustered DNA damage yields in irradiated cells by SFGE with the appropriate enzymatic treatments to detect the corresponding clustered base damage. SFGE indicated the fragmentation of chromosomal DNA in the irradiated cells based on the elution from the plug position on the electrophoretic gel, indicating DSB occurrences in the macromolecular DNA. In this analysis, the use of appropriate DNA glycosylases enabled us to assess clustered DNA damage yields, including oxidative pyrimidine and purine lesions based on EndoIII- and Fpg-sensitive sites, respectively [6]. At the D10 for cell survival, DSB damages (resulting from the digestion at the EndoIII- and Fpg-sensitive sites, respectively [6]. At the D10 for cell survival, DSB damages (resulting from the digestion at the EndoIII- and Fpg-sensitive sites) basically decreased as LETs increased (Fig. 2). The damage yields showed a decreasing tendency as LETs increased; gamma-rays > carbon > silicon > argon > iron. This relationship between DNA damage yields and LETs was similar to our previous result [6]. The decreasing slopes of the damage yields were sharp in the lower LET region, and the differences in the damage yields were little in the higher LET region. This suggests that the quantity of clustered DNA damage is unlikely to affect the RBE directly.

Next, we analyzed the isolated DNA damages using the ARP procedure. ARP specifically binds AP site at the C1-aldehyde site of the deoxyribose, losing its N-glycosidic base, and it works as a labeling agent for the AP site [23]. As mentioned above, the AP site is also the reaction intermediate in the BER process, and an appropriate DNA glycosylase can function to reveal the corresponding base lesion [6, 21]. EndoIII- and hOGG1-sensitive sites are nearly identical to oxidative pyrimidine and purine lesions, respectively, and both include AP sites. The modified ARP procedure showed that CHO-AA8 cells irradiated by gamma-rays presented a certain number of AP sites (11.1 × 10^−8 bp), EndoIII-sensitive sites (20.1 × 10^−8 bp), and hOGG1-sensitive sites (14.8 × 10^−8 bp) at the D10 for cell survival (Fig. 3). These base lesion yields are identical to those of the HeLa cells irradiated by 60Co gamma-rays reported in our previous study [21], indicating that our experimental conditions were appropriate. The respective base lesions decreased as LETs increased (gamma-rays > carbon > silicon > argon > iron), as observed for clustered DNA damages, also confirming our previous result [6]. Thus, the quantity of isolated oxidative base lesions does not seem to be an important factor for the RBE of heavy ion beams.

As mentioned above, another key factor involving clustered DNA damage must be identified in order to understand the heavy ion beam effect. To date, there is no effective technique to analyze the inner structure of clustered DNA damage experimentally, but a simulation methodology is available [7]. Therefore, we here investigated DNA repair for clustered base damage after heavy ion beam irradiation for this purpose. Among multiple DNA repair pathways, we focused on BER, because this repair pathway is mostly responsible for oxidative base lesions, including isolated and probably clustered ones. Xrcc1 is a
scaffold protein for active LigIII function. CHO-EM9 lacks functional Xrec1, and this results in no LigIII activity [13]. Thus, LigIII cannot repair the DNA by sealing the strand gap during the BER final step without Xrec1, resulting in the lack of completion of the short patch subpathway in EM9 cells [24]. Therefore, the BER intermediate strand break is retained in the mutant cell. If the repair-targeted base lesions are gathered (clustered base damage), DSBs will be retained after the incomplete BER activity without the functional ligation step. We compared the post-irradiated DSB accumulation in the wild type and the Xrec1 mutant CHO cells by SFGE. The wild type AA8 cells showed no accumulation of DSBs as the result of repair completion of clustered base damage induced by both gamma-rays and carbon ion beams in the post-irradiation culture (Fig. 4B and C). The mutant EM9 cells lacking Xrec1 accumulated DSBs after irradiation by carbon ion beams (Fig. 4E and F). The yield of additional DSBs increased with dose elevation. The accumulation was thought to be due to incomplete repair of clustered base damage. Since this DSB addition might be derived from the enzymatic process of clustered base damage, the accumulation appeared 1–3 h later. The mutant strain showed no DSB accumulation after gamma-irradiation (Fig. 4E and F). These results suggest that the damage can be processed by BER, leading to the induction of DSBs as the intermediate byproduct after carbon ion irradiation, but not gamma-rays (effectively). The sensitivities of these two strains to gamma-rays and carbon ion beams were also assessed. The mutant strain without Xrec1 was more sensitive to both ionizing radiations (Fig. 5). The increment of sensitivity for carbon ion beams shown by the mutant was larger than the wild type. This seems to link a relationship between the sensitivity and the DSB accumulation in the mutant cells, as previously described (Fig. 4E and F). It suggests that DSB derived from clustered base damage shows a more malignant behavior than the original clustered base damage via the BER process.

In contrast, the defective enzymes involved in another BER step showed a different result. A mutant Mef cell lacking both Nth1 and Ogg1 were used to analyze the function of the BER DNA glycosylase step in clustered base damage repair. The colony-formation assay indicated that the mutant Mef cell lacking both DNA glycosylases for oxidative base lesions became more resistant to gamma-rays and carbon ion beams (Fig. 6). Also, the mutant cells showed no accumulation of DSBs in the post-irradiation, unlike the CHO mutant (data not shown). These results indicate that the DNA glycosylase activity involved in the BER pathway can convert relatively benign clustered base damage to malignant DSB. The mutant cell's radio-resistance to carbon ion beams was higher than that to gamma-rays. It seems to be connected with later additional DSBs as secondary products derived from the process of clustered base damage.

**DISCUSSION**

Heavy ion beams are more detrimental to any living organisms than the commonly used gamma-rays or X-rays. Thus, the use of heavy ion beams is expanding in a wide range of areas, such as cancer therapy. However, we have less information about the effects of heavy ion beams and their underlying molecular mechanisms. We previously observed that yields of the damage induced by heavy ion beams decreased as LET's increased in a test tube study [6]. To understand the biological impact of DNA damage (especially clustered DNA damage as a specific radiation damage), we first analyzed the cellular clustered DNA damage induced by heavy ion beams. Our SFGE results show that clustered DNA damage yields at the D_{10} for cell survival declined gradually as the LET increased (Fig. 2). This inverse relationship between damage yields and LET has been observed in other studies [7]. LET is an inverse function of the flux of radiation. Briefly, LET increase is equal to the flux decrease. Radiations with lower flux have fewer occasions to encounter the target DNA molecule, and might result in smaller yields of damage. Our present results for both clustered and isolated damages fit with this concept (Figs 2 and 3). Additional mechanisms for the decrease in DNA damages are differences in the ionization rate, and proportion of direct and indirect effects between higher and lower LET radiations. The primary products from H_{2}O, such as hydroxyl radical (·OH) and hydrogen radical (H·), decrease as LET increases [25]. At higher LET radiations, these primary radicals are quickly consumed to generate the secondary products such as hydrogen peroxide (H_{2}O_{2}), leading to the loss of more active species attacking the DNA, and, therefore, to a decrease in DNA damage yields. The short-lived active species such as ·OH quickly meet each other inside a more condensed track of high LET radiation, and they are hard to diffuse far from their origin point [26]. Thus, they cannot attack the DNA molecule efficiently, resulting in less damage. Previous studies indicated that the direct damaging action of ionizing radiations increased as the LET increased [27, 28].

On the other hand, simulation studies indicated that higher LET radiations more effectively generate clustered DNA damages [7]. In
the present study, we showed that clustered DNA damage yields per flux increased as the LET increases. Our SPGE results suggest that higher LET radiations generate DNA damages per flux more effectively than lower LET radiations. We can estimate that iron ion beams present clustered DNA damage yields that are 100 times greater than those of gamma-rays on a flux basis. Despite their abilities to generate higher amounts of clustered DNA damage, the yields of damage decrease in the higher LET range in this study. This might be due to the exponential decrease in the flux with the LETs.

Our present results indicate that clustered DNA damage yields decrease as LET increases, but the respective D_{10} of these irradiated surviving cells apparently follows the LET elevation (Fig. 1). Thus, the biological effect of heavy ion beams must involve other factors than the quantity of clustered DNA damage. Here, the isolated DNA damage is excluded, because the yields decrease more rapidly for LET than for the clustered ones (Figs 2 and 3). In this study, we are discussing clustered DNA damage in connection with LET. On the other hand, we know lineal energy (γ) as another index from the microdosimetric approach. For high-LET radiation (such as heavy ion beams), γ is thought to be more suitable for discussing the RBE. However, we have less information on γ for the radiations and cell species we used. We examined our present result with γ (from a study covering some of our radiations but for other cell line) [29], and we found a similar relationship to that for LET. We need further detailed information from rigorous microdosimetric studies conducted under the experimental conditions that we used in order to understand the biological effect of heavy ion beams more precisely.
While the simulation studies for clustered DNA damage induced by heavy ion beams progress, we have no experimental procedure for analyzing its complexity as yet. In this study, we opted for another approach, involving DNA repair, to elucidate the effect of clustered DNA damage induced by heavy ion beams. We previously indicated that the purified Endo III and Fpg can process their target base lesions in cluster forms, and that clustered base damage can be converted to DSB using the model oligonucleotide substrates (including bistranded thymine glycols and 8-oxoguanines, respectively) [6]. Both Endo III and Fpg effectively generated DSBs from the respective clustered base damages when the two opposite base lesions were set from over three nucleotides apart. Similar results were observed for synthesized clustered base damages with the corresponding purified DNA glycosylases [12, 30, 31]. It is reported that a clustered AP site was converted to DSB by enzymatic activity in a test tube [32, 33]. Our present result indicates that DNA glycosylases involved in the BER process of clustered base damages affect biological consequences in cells irradiated by heavy ion beams. The BER process starts with the removal of inappropriate bases, including base lesions, by DNA glycosylases [9]. DNA glycosylases remove the corresponding base lesion and an AP site is generated at this position. Next, the AP endonuclease incises the DNA strand at the AP site. A certain type of DNA glycosylase (such as...
NTH1 or OGG1) subsequently incises the DNA strand through its AP lyase action [34, 35]. When any AP endonucleases act, base lesion in the DNA is once converted to DNA strand break during the BER process. If a set of base lesions is located three nucleotides apart, the DNA glycosylase activity is inhibited [6, 10–12]. It indicates that clustered base damage consisting of multiple oxidative pyrimidines and/or purines that stand on the respective strands and over three nucleotides apart from each other can be converted to DSB by the BER process. There is no conversion from clustered base damage to DSB in cells lacking both NTH1 and OGG1, resulting in no accumulation of DSBs post-irradiation, as mentioned above. The mutant cell lacking both DNA glycosylases showed an increased resistance to ionizing radiations when compared with the wild type cell (Fig. 6). This result indicates that clustered base damages were really converted to DSBs in the post-irradiation culture, revealing a more malignant potential as DSBs. Cell survival was also greater for mutant cells irradiated with carbon ion beams than for those irradiated with gamma-rays, when compared with the wild type. It suggests that clustered base damage induced by carbon ion beams is more complex than that induced by gamma-rays. High-LET radiation (such as heavy ion beam) has larger track structure with a more condensed ionizing region around it. It suggests that the constituent lesions (SSBs and respective base lesions) might exist to concentrate in clustered DNA damages. The densely existing lesions in clustered DNA damage will be more easily processed to DSB. DSB shows higher cytotoxicity than clustered base damage among clustered DNA damages [36], and this damage has been thought to be the most important life-threatening damage. But base damage, including its cluster, is likely to be involved in mutagenesis [37]. Overexpression of those DNA glycosylases resulted in increased cell sensitivity, mutation frequency, and increase in DSB generation in post-irradiation cell culture [38, 39]. Our present result confirms these previous results for the BER process of clustered DNA damage.

Mutant cells lacking Xrc1 (which reduces their ligation activity with LiggI [13]) was more sensitive than the wild type cell (Fig. 5). The mutant also presented an increased sensitivity to carbon ion beam radiation compared with the wild type cells in the lower dose range. It indicates that higher-LET radiations induce more complex damage, leading to DSB generation, in agreement with simulation studies [7]. Only the mutant cell accumulated DSBs after irradiation with carbon ion beams, but not with gamma-rays (Fig. 4). The result with BER-mutant cells suggests that the cellular processing of clustered base damages leading to DSB addition affects the biological consequence of heavy ion beam irradiation [37]. The BER process for clustered base damage is an important factor for heavy ion irradiation effects, like other DNA repair functions such as homologous recombination (HR) and non-homologous end joining (NHEJ). Additionally, a recent study indicated that the activity of Apel, a mammalian AP endonuclease, leads DSB increments after high-LET irradiation, and is involved in the sensitivity of irradiated cells [40]. Thus, the early glycosylase and AP endonuclease steps can increase DNA strand breaks as their reaction intermediates, and then the last ligation step can eliminate the strand scissions. Therefore, incompleteness of the last step leads to DSB accumulation (Fig. 4E and F), and results in a more malignant effect for the cells (Fig. 5).

Of course, we recognize that post-irradiated DSBs is caused by other pathways. DSB is a result of apoptosis in irradiated cells. A previous study indicates that apoptotic DNA fragmentation begins to increase 12 h-post-irradiation and becomes maximal from 36 to 60 h for CHO cell lines (including AA8), which have been irradiated by gamma-rays (1–16 Gy) [41]. Our 3 h-post-irradiation culture period is inadequate to investigate apoptotic DSBs. It suggests that the post-irradiated DSB increments in the present study include no apoptotic DSBs. Also, we need to consider that DSBs arise with cell proliferation. A study for PARP inhibitor effect on the radiation damage process indicates that the PARP-dependent DSBs increase does not appear within the first 5 h after irradiation [36]. Although that result is from a different cell line, our result of DSBs within 3 h after irradiation might eliminate replication-associated DSBs. Also, another study indicates that Chinese hamster–derived cells demonstrate that the average proliferation time is delayed instantaneously by at least a factor of two with the cell cycle arrest [42]. This confirms our conclusion, as mentioned above. Therefore, DSB accumulations after irradiation are derived from repair intermediates of clustered base damage by BER, as investigated in the present study.

In conclusion, clustered DNA damage yields (including DSBs and clustered base damages) decreased as the LET increased in cells irradiated by heavy ion beams. This result leads to an expansion of our previous test tube result to the cellular event [6]. The consistency observed between the damage yields and the RBE seems to be achieved by clustered base damage processing in irradiated cells. Although we featured BER in this study, we know that other repair pathways, such as HR and NHEJ, are involved in DSB repair [37]. We also consider that experimental procedures should be established to analyze the complexity of clustered DNA damage induced by ionizing radiations. For instance, an electrophoresis procedure such as SFGE, used in this study, cannot be used to assess the inside of multiply damaged sites, namely the inner structure of clustered DNA damage [43]. We experimentalists are considering further effort to understand the biological effect of heavy ion beams more precisely, as with the above-mentioned requirements, and also more close collaboration with simulation studies.

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