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LET Dependence of 8-Hydroxy-2′-deoxyguanosine (8-OHdG) Generation in Mammalian Cells under Air-Saturated and Hypoxic Conditions: A Possible Experimental Approach to the Mechanism of the Decreasing Oxygen Effect in the High-LET Region

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One of the most distinguished features in biological effects of heavy ions would be the decrease of oxygen effect in the high-LET region. This feature has been referred to as the radiobiological basis for the control of hypoxic fraction in cancer radiotherapy. However, mechanisms to explain this phenomenon have not been fully understood. One of the explanations was given by the oxygen in the track hypothesis, which proposes that oxygen is produced along ion tracks even in the hypoxic irradiation condition. In the present study, we designed an experimental approach to support this hypothesis by using 8-hydroxy-2′-deoxyguanosine (8-OHdG) as DNA damage requiring oxygen to produce. The LET dependence of 8-OHdG under hypoxic condition revealed that with increasing LET 8-OHdG yield seems to increase, despite that the yield of OH radical, which is also required for the production of 8-OHdG, decreases in the high-LET region. This result is consistent with the explanation that the local generation of oxygen along ion tracks contributes to the increase of 8-OHdG yield.

INTRODUCTION

Cancer radiotherapy using heavy ions has been expected to control hypoxic fraction because of its high-linear energy transfer (LET) nature, based on the phenomenon that oxygen effect decreases with increasing LET. The LET dependence of oxygen enhancement ratio (OER) particularly in terms of cell death has been extensively studied as typically summarized in the references (*1–7*). However, mechanisms to explain this phenomenon have not been fully understood. Two major possible mechanisms were proposed as "interacting radical hypothesis" (*8*, *9*) and "oxygen in the track hypothesis" (*10*) around the middle of the 1900s. According to the interacting radical hypothesis, as the LET increases, the number of radical lesions is decreased due to the increased reactions among closely generated radicals followed by the decrease of indirect action where radical lesions are predominant, resulting in the decrease in the requirement of oxygen to fix radical lesions. This model was later extended by considering multiple radical sites (*11*). On the other hand, according to the oxygen in the track hypothesis, oxygen is produced along heavy-ion tracks as a result of the radiolysis of water distinctive of high-LET radiation even in the irradiation under hypoxic condition, which leads to decreased difference in radiobiological effects between under oxic and hypoxic conditions. Baverstock and Burns measured G value of O_2 in the acidic solution and discussed the relation to the oxygen effect (*12*), while Sauer et al. raised a doubt at this model by considering the significant diffusion of oxygen from track cores with the measurement of oxygen yield under neutral condition (*13*). In the following study by Baverstock and Burns (*14*), they explained the LET-OER relationship based on the oxygen in the track model, by the simulation study considering the oxygen diffusion process from track cores. Although several studies suggested that the oxygen in the track model is unlikely to be applied to the explanation of the decrease of oxygen effect (*15*, *16*), recent quantitative evaluation of local oxygen concentration along a beam track using Monte Carlo simulation would be a strong support for the oxygen in the track hypothesis (*17*, *18*). In these studies, the oxygen concentration in the micro-second range after irradiation, in which

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lesions to biomolecules are supposed to be fixed by oxygen, was estimated at a few micro molars, which agreed well with the oxygen concentration required to express oxygen effect (*19*). The recent advance in radiotherapy, ultra-high dose-rate pulsed (FLASH) method, is going to be planned to extend to particle radiotherapy. Although the exact mechanism underlying FLASH method is obscure, the oxygen production in the high-dose rate region around Bragg peak is discussed as a possible explanation to the effectiveness of FLASH method (*20*).

To provide possible experimental basis in favor of the oxygen in the track hypothesis, we planned to examine the production of oxygen dependent DNA damage under hypoxic irradiation condition. We adopted a popular guanine oxidative damage, 8-hydroxy-2′-deoxyguanosine (abbreviated as 8-OHdG), because it requires oxygen to produce (*21–23*) and the analytical method to measure 8-OHdG has been established and is easily available using HPLC coupled with an electrochemical detector (abbreviated as HPLC-ECD system). The participation of oxygen in the 8-OHdG generation is supposed as follows: Guanine in DNA or double-stranded oligonucleotides reacts with OH radicals to form guanine radical cation ($G^{\bullet+}$) as a result of one-electron oxidation (*24*). Subsequent hydration of $G^{\bullet+}$ leads to the formation of 8-hydroxyl radical adduct (8-OH-G●) (*24*), which readily reacts with oxygen at diffusion-controlled rate with a rate constant of 4×10^9 s^{-1}, resulting in the generation of 8-OHdG (*25*, *26*). Another pathway for the direct generation of 8-OHdG from DNA or double-stranded oligonucleotides via pyrimidine peroxyl radical also requires oxygen (*24*).

In our previous study, we measured LET dependence of 8-OHdG generation in 2′-deoxyguanosine (abbreviated as dG) aqueous solution irradiated with heavy ions under air-saturated and hypoxic conditions (*23*). Although 8-OHdG yield decreased with increasing LET due to the decrease of the yield of OH radicals that are one of the major species for the production of 8-OHdG in both irradiation conditions, the decrease was not so significant in the case of hypoxic condition compared with air-saturated condition. These results may be interpreted as that the decrease under hypoxic condition is partly suppressed by the additional production of 8-OHdG caused by the production of oxygen along ion tracks. Monte Carlo simulation was also conducted to calculate LET dependence of 8-OHdG generation for oligonucleotides in liquid water (*27*). The yield of single isolated 8-OHdG decreased with increasing LET, which was in accord with the results of the dG solution system. However, in contrast to the case of single 8-OHdG, the yield of complex damage containing 8-OHdG increased with increasing LET, suggesting that we should pay attention to a possible generation pathway of 8-OHdG unique to high-LET radiation.

The present study is the extension of our in vitro solution study to the cellular system using mammalian cells, which is much more proper system to examine the cellular oxygen effect. In addition, since in the diluted solution system, it is thought that the spatial arrangement between ion tracks and homogeneously distributed dG molecules of low concentration may significantly lower the yield of 8-OHdG, while the cellular system with rather condensed structure of nucleic acids in the nuclear region would be expected to give clearer results.

TABLE 1
Nuclides and LETs of Heavy Ion Used

Nuclide	Injection energy (MeV/u)	LET in the middle of cell suspension (keV/μm)	LET range in 1-mm thick cell suspension (keV/μm)
Carbon	290	13	13–13
($^{12}C^{6+}$)		20	20–20
		50	48–52
		80	71–95
		100	82–151
Neon	400	80	79–82
($^{20}Ne^{10+}$)		150	140–170
		200	176–269
Silicon	490	55	55–55
($^{28}Si^{14+}$)		80	79–81
		150	147–153
		200	188–213
		250	234–273
Iron	200	440	440–440
($^{56}Fe^{26+}$)		736	682–797

MATERIALS AND METHODS

Cell Culture and Sample Preparation for Irradiation

Human leukemia, HL-60 cells (*28*) were cultured in an exponential growth phase in an RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were washed with phosphate buffered saline (PBS) twice, and were finally concentrated at the density of 2.5×10^7 cells/ml suspended in PBS.

Irradiation with Heavy Ions and X rays

Heavy ions were supplied by the Heavy Ion Medical Accelerator in Chiba (HIMAC) at QST in Japan. Table 1 summarizes the ion nuclides, injection energies, and LETs used. The LET in the middle of the sample chamber for cell suspension, which has a space of 1 mm thick and is perpendicular to beam direction, is the representative of the LET range over the sample chamber (the fourth column of Table 1). On the contribution of ion fragmentation, the radiation dose is still delivered mainly by primary ions, with a contribution of less than about 20% from fragmented lighter ions, in spite of the decreased number of primary particles to less than 50% (*29*). For the irradiation under air-saturated condition, the cell suspension was poured in the sample chamber designed for solution samples made of PMMA, which is described previously (*23*). To achieve hypoxic irradiation condition, cell suspension in a flask was flushed with humidified gas mixture of 95% N_2 and 5% CO_2 for 1 h at flow rates of 300 ml/min and 15 ml/min for N_2 and CO_2, respectively. The hypoxic cell suspension was then introduced into a specially designed irradiation sample chamber made of glass for the front and stainless steel for the back. The replacement of air to N_2 and CO_2 was checked by giving an OER value of about 3 in the case of cell killing by X-ray irradiation as described previously. This OER value ensured that radiobiological hypoxia was routinely achieved, indicating that oxygen levels were below 0.7 mmHg (about 0.1% oxygen) (*23*). The irradiation was performed at 4°C to suppress possible repair of 8-OHdG. The radiation dose was usually adopted as 300 Gy and 600 Gy for the air-saturated and the hypoxic condition, respectively, except for experiments to obtain

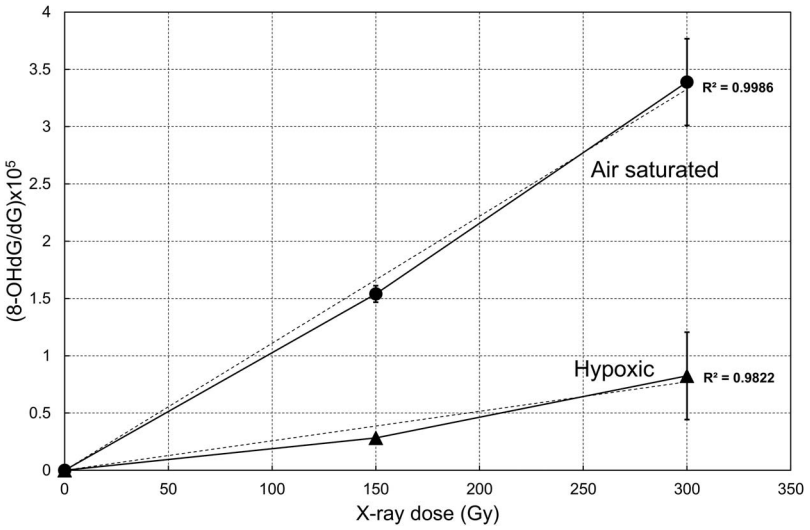

FIG. 1. Dose dependence of 8-OHdG generation irradiated with X rays under air-saturated and hypoxic condition. Solid circles: air-saturated condition, solid triangles: hypoxic condition. The broken lines show regression lines and the coefficient of determination is also shown for the evaluation of the linear relationship.

a dose dependence relationship. The irradiation was repeatedly conducted more than 3 times for every LET and ion species.

X-ray irradiation was performed with an X-ray generator with the voltage of 58 kV (SOFTEX Co., Japan). The LET of X rays was estimated at approximately 6 keV/μm (*30*).

8-OHdG Extraction from Mammalian Cells

DNA was extracted from HL-60 cells using DNA Extractor® TIS Kit (Wako Pure Chemical Industries, Ltd., Japan), because this kit has an advantage to suppress an increase of background oxidation by employing the NaI extraction method and by adding an antioxidant agent. NaI has been reported to suppress the control level of 8-OHdG (*31*). For the isolated DNA, 8-OHdG was further extracted by digesting heat-denatured DNA to nucleotides with Nuclease P_1 (Wako Pure Chemical Industries, Ltd., Japan) and subsequent digestion with Alkaline Phosphatase (E. coli C75; Takara Bio Inc., Japan) to obtain nucleoside mixture. After the filtration with Ultra free® Centrifugal Filters (0.22 μm; Millipore Corp., MA) or VIVASPIN 500 (Sartorius Stedim Biotech, Goettingen, Germany), samples were ready for HPLC analysis.

The above-described procedure was chosen for the following experiments through the comparison with a conventional DNA extraction method using 2-propanol or a method using the previous version of DNA extraction kit, named as the WB kit, manufactured by Wako Pure Chemical Industries. The propanol method and the WB kit measured about 3.5 times and 1.4 times higher amount of 8-OHdG for unirradiated control sample, indicating that the TIS kit significantly suppresses the undesirable oxidation of guanine residues during the extraction of 8-OHdG.

8-OHdG Separation by HPLC

The extracted solution was applied to an HPLC system (Shimadzu Corp., Japan) coupled with Coulochem III (ESA) for the electrochemical detection of 8-OHdG. The total amount of 2′-deoxyguanosine (dG) in the solution was detected with UV detection using a photo-diode array detector (SPD-M10A_{VP}, Shimadzu Corp, Japan). The solution was injected into an HPLC column (TSKguardgel ODS-80Ts, Tosoh Corp., Japan) with organic solvent mobile phase consisting of 10 mM NaH_2PO_4 in 8% MeOH. The amounts of 8-OHdG and dG were calculated using values obtained with the HPLC analysis of standard solutions of 8-OHdG (Sigma-Aldrich) and dG (Sigma-Aldrich) of known concentrations. The 8-OHdG amount was expressed as 8-OHdG molecules produced per dG molecules. The control value for an unirradiated sample was around 0.5 8-OHdG/10^5 dG.

RUSULTS AND DISCUSSION

Dose Dependence of 8-OHdG Generation in X ray or Carbon-Ion Irradiated Cells

Figure 1 shows dose dependent induction of 8-OHdG by X rays under air-saturated and hypoxic conditions. Data in which the control value was subtracted were plotted. Both graphs appear to show almost linear relationship at least in the dose range tested, judging from the inserted dotted regression lines and coefficients of determination, which was in agreement with the previous reports for DNA solution (*21*, *32*), mammalian chromatin (*22*), and mouse liver (*33*). While the linear increase of 8-OHdG yield was observed at least in the dose up to 200–300 Gy (*21*, *22*), at the higher doses the yield tends to show a saturation curve. For example, in the literature (*32*) at the dose of 600 Gy the curve began to be convex upward, although in the experiments dealing with the lower level of 8-OHdG yield, the saturation does not appear to be reached yet. The saturation tendency may result from successive formation of oxidation products after 8-OHdG generation (*24*, *34*, *35*). However, there is a report that showed a linear response up to 1,000 Gy for carbon-ion irradiated mammalian cells (*36*). Since high-LET radiation induces less amount of 8-OHdG, the saturation dose may be shifted to the larger dose region.

The irradiation under air-saturated condition induced much larger amount of 8-OHdG as expected from the previous studies (*21*, *22*). It would be interesting to compare the 8-OHdG yield ratio (yield in air-saturated condition/yield in hypoxic condition) among specimens for irradiation. For DNA solution the value is almost infinite, because 8-OHdG in hypoxic condition was not detectable (*21*). In the case of chromatin solution,

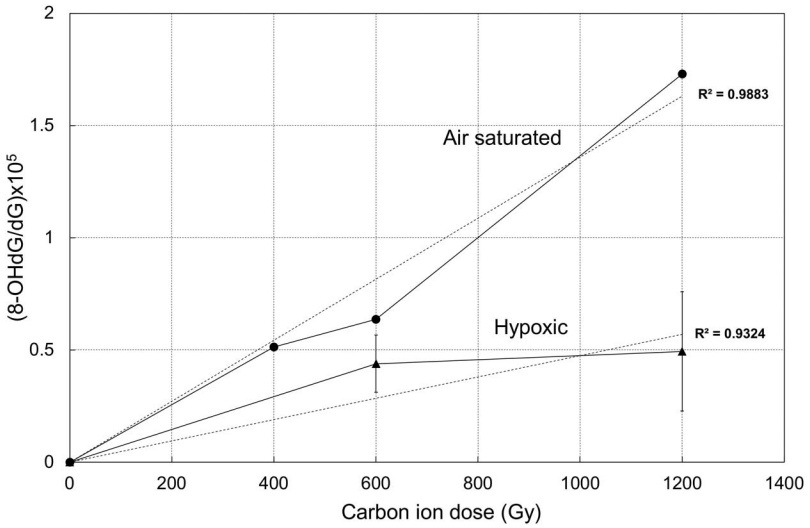

FIG. 2. Dose dependence of 8-OHdG generation irradiated with carbon ions with an LET of 100 keV/μm under air-saturated and hypoxic condition. Solid circles: air-saturated condition, solid triangles: hypoxic condition. The broken lines show regression lines and the coefficient of determination is also shown for the evaluation of the linear relationship.

the value is approximately 4.5 (*22*), and in our mammalian cell case, the value decreased to 4.1. In addition, for double-stranded oligonucleotides, the value of 3.4 was nearly similar to our results (*24*, *37*). The different reaction pathways of OH radical adduct of DNA with surrounding molecules in different environments have been discussed (*24*, *38*). It should be added that these previous studies employed ^{60}Co γ radiation, an LET of which is 0.2 keV/μm (*39*), with more uniform spatial distribution of energy deposition, compared with our X-ray source with an LET of about 6 keV/μm. However, the above similar value of the 8-OHdG yield ratio to that obtained with ^{60}Co γ source suggests that our X-ray apparatus could be used for the same purpose as a typical low-LET ^{60}Co γ source.

The results for carbon-ion irradiation with an LET of 100 keV/μm were shown in Fig. 2 as an example of heavy-ion irradiated specimens. The amount of 8-OHdG was significantly decreased compared with the case of X-ray irradiation for both oxygen conditions. The dose-dependence curves exhibited further convex upward compared with X rays, because the dose range was extended up to 1,200 Gy, compared with X-ray experiments. This observation was understood from a smaller value of the coefficient of determination under the assumption of the linear regression. Note that the difference between air-saturated irradiation and hypoxic irradiation became smaller than the case of X-rays, which may result from oxygen production in the high-LET region as suggested by oxygen in the track hypothesis and/or possible increasing participation of an oxygen free pathway in the 8-OHdG generation as discussed later.

LET and Ion Species Dependence of 8-OHdG Generation In Heavy-Ion Irradiated Cells

To secure a significant 8-OHdG yield and to achieve irradiation with various LETs and ion species in limited beam time, we determined a single representative dose to compare the yield between different LET and ion species. A dose of 300 Gy and 600 Gy was adopted for air-saturated and hypoxic condition, respectively, since these doses seem to be an upper limit in the approximate linear relationship. Figure 3 shows LET dependence under air-saturated condition for carbon, neon, silicon and iron ions. It is of interest to compare with the previous study on the 8-OHdG generation after irradiation with heavy ions. Pouget et al. found twofold lower yield of 8-OHdG in mammalian cells after exposure to carbon ions with an LET of 25 keV/μm than after exposure to γ radiation (*36*). This result is in good agreement with our results in Figs. 1 and 3; from Fig. 3, 8-OHdG amount was about $1.7/10^5$ dG/300 Gy at an LET of around 25 keV/μm, while in Fig. 1 for X rays, 8-OHdG was about $3.4/10^5$ dG/300 Gy, the ratio of which well corresponds to that obtained by Pouget et al.

To explain the LET dependence of 8-OHdG generation, for reference, G values of OH radicals measured at the same irradiation facilities, HIMAC by Yamashita et al. (*40*) were inserted in the same graph. With decreasing G value of OH radicals with increasing LET, the 8-OHdG yield seems to decrease in parallel at least up to around 100 keV/μm, as particularly observed for carbon and neon ions. However, above about 100 keV/μm, in contrast to decreasing tendency of G value of OH radicals, the decrease of 8-OHdG yield was not so significant: For neon, silicon and iron ions, the yields were nearly constant or in some cases increasing with increasing LET. LET dependence of G-value of oxygen, which was obtained by the calculation using Monte Carlo simulation incorporating multiple ionizations at 10^{-6} sec after $^{12}C^{6+}$ irradiation (*17*), was also plotted in the same graph. Significant increase of oxygen yield was observed above 100 keV/μm. These may be resulted from the balance between decreasing OH

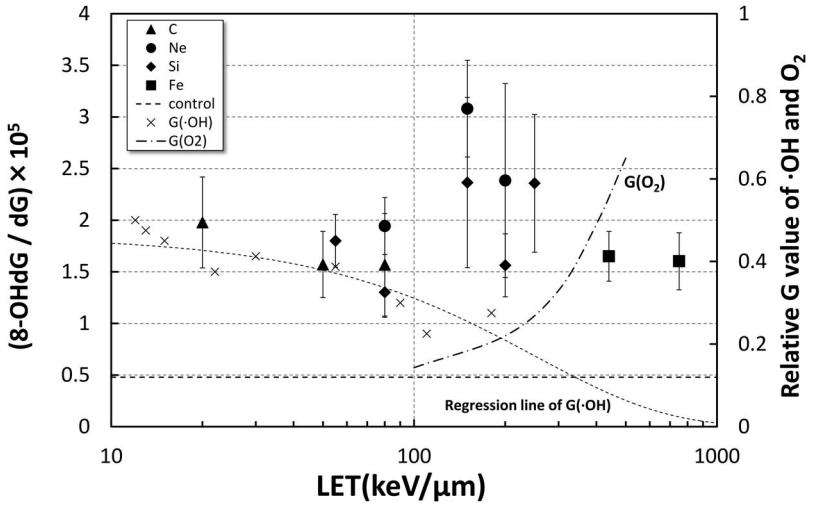

FIG. 3. LET dependence of 8-OHdG generation irradiated with various ions under air-saturated condition. Ion species include carbon, neon, silicon, and iron. 8-OHdG values obtained at 300 Gy are shown. G value of OH radicals reported by Yamashita et al. (*40*) and G value of oxygen reported by Meesungnoen et al. (*17*) are plotted together. Broken line shows the level of unirradiated sample.

radical yield and the generation of oxygen in the high-LET region. Furthermore, these results suggest that the contribution of local oxygen production along radiation tracks is more significant compared with evenly distributed oxygen in cell suspension. We could interpret that 8-OHdG is detected at a high concentration on the heavy-ion track and the oxygen concentration becomes comparable or higher than that in cellular environment.

The above explanation becomes more plausible in the experiments under hypoxic irradiation. In contrast to the case of the air-saturated condition, 8-OHdG generation is thought to be mainly limited by the oxygen concentration rather than the OH radical yield. Therefore, if oxygen is produced along radiation tracks with increasing LET, the increase of 8-OHdG with LET is expected to be more clearly observed in spite of the decreasing OH radical yield. The results shown in Fig. 4 agree with our expectation. In fact, the data of carbon ions show that even if OH radicals are produced, the yield of 8-OHdG is quite low in the absence of oxygen (Fig. 4). In the case of silicon and neon ions, although the yield of OH radicals decreased in the high-LET region, the yield of 8-OHdG was significantly higher than that in the lower LET region (below 100 to 200 keV/μm), which is consistent with the increasing G-value of oxygen. In the very high-LET region that was achieved by using iron ions,

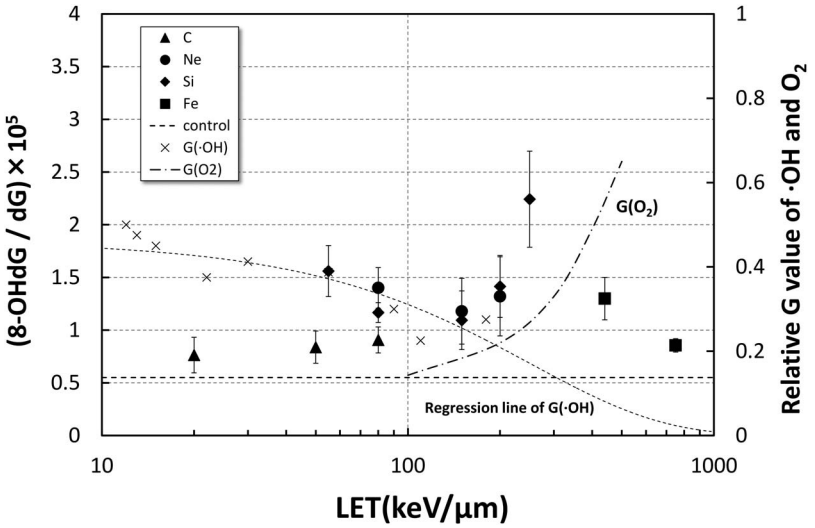

FIG. 4. LET dependence of 8-OHdG generation irradiated with various ions under hypoxic condition. Ion species include carbon, neon, silicon, and iron. 8-OHdG values obtained at 300 Gy are shown. G value of OH radicals reported by Yamashita et al. (*40*) and G value of oxygen reported by Meesungnoen et al. (*17*) are plotted together. Broken line shows the level of unirradiated sample.

the yield was somewhat decreased with increasing LET, which may be due to the very small yield of OH radicals in such very high-LET region. It should be emphasized that these experimental results particularly obtained in the hypoxic condition are consistently interpreted by the oxygen in the track hypothesis.

In addition, the variation of 8-OHdG yield seems to be large particularly for the air-saturated experiments compared with the hypoxic experiments. Therefore, the experiments were repeated from 5 to 20 times for each irradiation condition. Although the cause of such unstable yield in the air-saturated case is obscure, the similar situation was reported previously (*41*). Incidentally the control level in the hypoxic condition illustrated as the broken line was unexpectedly somewhat higher than that in the air-saturated condition. Although the values were within the error range, it is possible that hypoxic stress induces oxidative stress, which raises the level of cellular 8-OHdG. However, since considerably different response to high-LET radiation in both oxygen conditions was observed in Figs. 3 and 4, it is unlikely that the hypoxic stress induces such a complex behavior depending on LET.

Comparison of 8-OHdG Generation in the Cell System and in the Deoxyguanosine Solution System

We have reported the similar experiments of LET dependence of 8-OHdG generation in dG aqueous solution system (*23*). In the in vitro experiments, 8-OHdG yield decreased with LET increase nearly in parallel with the decrease of OH radial yield in the air-saturated condition, while in the hypoxic condition the decrease of 8-OHdG was much less than in the case of the air-saturated condition. However, in contrast to the present cellular system, the increasing tendency of 8-OHdG yield with LET was not observed. We cannot specify the cause of the clearer results obtained in the cellular system, but possible explanations would be as follows: 1. difference in reaction probability between diffusing molecules, and 2. different reaction pathway leading to 8-OHdG generation in different molecular environment of DNA bases. Concerning the first possibility, since in the cellular system DNA region through which ion particles pass in the nucleus is immobilized in the inner nuclear structure, DNA is present in the vicinity of the oxygen produced along ion tracks, resulting in giving the clear participation of oxygen. For instance, while the dG concentration in aqueous solution was 0.5mM in the in vitro experimental condition (*23*), the concentration of intra-nuclear molecules such as chromatin was estimated to be of the order of 1M (*14*), which was 10^4 times higher than the solution experiment. Furthermore, the diffusion constant of highly polymerized biomolecules in a nucleus was estimated as 10^5 to 10^6 times smaller than dG in solution from the calculation based on the formula in the reference (*42*), which means that nucleus molecules are nearly immobilized. These considerations suggest that the probability of the reaction of highly concentrated and immobilized guanine molecules with oxygen produced along ion tracks in the vicinity of DNA would be much higher than the reaction between freely diffusible dG and locally produced oxygen. In addition, it is pointed out that the reaction probability between two diffusible components of guanine and oxygen becomes much decreased (*13*). Thus the participation of oxygen generated along ion tracks could be more clearly detected in the cellular situation.

In favor of the second possibility, there have been several reports to raise a problem that OH radical reactions with DNA or its components are strongly dependent on the molecular environment (*24*, *38*). In the case of dG, the major reaction pathway of OH radicals with dG is the guanyl radical G(-H)• formation with less contribution of the direct addition of OH radicals to dG, which leads to the formation of 8-OH-G•, a precursor of 8-OHdG (*24*), but in the case of double-stranded oligonucleotide, which is closer to the cellular DNA situation, 8-OH-G• is formed mainly via guanine cation radical followed by hydration reaction, not via guanyl radical (*24*, *35*). In addition to the pathway from 8-OH-G•, the direct pathway from double-stranded oligonucleotides via pyrimidine peroxy radical also requires oxygen as mentioned in the introduction (*24*), which strongly suggests the significant role of oxygen in the 8-OHdG generation in cellular environment. In support with this explanation, the reaction of DNA-OH radical adduct with oxygen is largely different between single-stranded or denatured DNA and double-stranded DNA (*38*). The authors found smaller yield of DNA peroxy adduct in double-stranded DNA, which may cause remarkable effect of oxygen, if produced inside DNA structure rather than spatially evenly distributed oxygen.

Evaluation of 8-OHdG Assay System for the Detection of Oxygen Participating DNA Damage

Using the cellular system, we further gave the additional reliable experimental evidence for the oxygen in the track hypothesis, compared with our previous trial of in vitro dG solution system. However, our 8-OHdG system to detect the presence of oxygen in the track has some uncertainty to provide the basis for the definite participation of oxygen: Firstly, in the high-LET region 8-OHdG production due to the produced oxygen tends to be hidden by the decrease of 8-OHdG due to decreasing G value of OH radicals. Therefore, the repeated measurements are definitely required, and we revealed the significant increase of the 8-OHdG production in the high-LET region for every kind of particles particularly in the hypoxic irradiation condition. Secondly, the possibility for the production of 8-OHdG in the absence of oxygen in the high-LET region should be discussed particularly in relation to direct action that is well known to increase in the high-LET region (*43*, *44*). Guanine cation radical $G^{\bullet+}$, a precursor of 8-OH-G•, can be formed from guanine through one-electron oxidation (*24*), partly by the participation of oxygen and/or OH radicals. In the high-LET region, there is an oxygen and OH radical free pathway leading to the formation of $G^{\bullet+}$ through hole transfer which may result from direct action (*45–47*). Sharma et al. proposed a reaction pathway for 8-OHdG formation by direct action

which is different from that occurs in bulk water (*48*). Since in the middle of this reaction pathway, guanyl radical G(-H)• is not so reactive with molecular oxygen (*26*, *49*), possible oxidants for the oxidation of G(-H)• are radical cations densely formed on nearby phosphate or deoxyribose (*48*). Furthermore, the increase of 8-OHdG produced inside complex cluster damage demonstrated by Monte Carlo simulation (*27*) appears to be consistent with that cluster damage induced by high LET radiation results mainly from direct action. This oxygen-free pathway by direct action could partly account for the smaller difference in 8-OHdG yield between air-saturated and hypoxic condition, compared with low-LET irradiation.

The next point is the possibility of the participation of oxygen-free pathway in the latter process from 8-OH-G• to 8-OHdG. It was proposed that the electron subtraction that is required in the formation of 8-OHdG from 8-OH-G• can be processed by surrounding guanine cation radicals in place of oxygen (*35*). Upon high LET irradiation, guanine cation radicals may be formed densely compared with low-LET X rays. This pathway for the generation of 8-OHdG without oxygen may also contribute to the increased generation of 8-OHdG in the high-LET region. The above-mentioned discussion favors the increase of reaction pathways to 8-OHdG without oxygen that mainly induced by direct action. However, concerning the absolute yield of 8-OHdG by such oxygen-independent pathways, note that 8-OHdG yield by direct action is decreased with increasing LET as well as that by indirect action, according to the study by Sevilla (*46*). They explained this results in terms of track structure of high-LET radiation which is generally classified into double structures: Core, a central part with high energy deposition and surrounding penumbra area consisting of δ-rays with low-LET nature. They described that base radicals produced by direct action are mainly from the penumbra area, and with increasing LET, fraction of beam energy deposited to penumbra is decreasing, resulting in the lower yield of base damage including 8-OHdG. Their discussion does not suggest that direct action alone leads to the apparent increase in the yield of 8-OHdG in the high-LET region observed in our results.

Although the presence of the above-described oxygen-free pathways should be considered, oxygen, if produced, is evidently an effective electron subtraction agent as shown by several literatures demonstrating the high production of 8-OHdG in the presence of oxygen. Again, it should be stressed that the increase of 8-OHdG from around an LET of 100 keV/μm was found to be nearly parallel with the increase of G-value of oxygen. Finally, our experimental study is consistent with the oxygen in the track hypothesis the validity of which has been discussed in the radiation chemical and simulation studies.

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