Exploration of ‘Over Kill Effect’ of High-LET Ar- and Fe-ions by Evaluating the Fraction of Non-hit Cell and Interphase Death

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Over Kill Effect/RBE vs LET Relation/Heavy Ions/Non-hit Cell/Interphase Death.

The reason why RBE for cell killing fell to less than unity (1.0) with very high-LET heavy-ions ($^{40}$Ar: 1,640 keV/$\mu$m; $^{56}$Fe: 780, 1,200, 2,000 keV/$\mu$m) was explored by evaluating the fraction of non-hit cell (time-lapse observation) and cells undergoing interphase death (calculation based on our previous data). CHO cells were exposed to 4 Gy (30% survival dose) of Ar (1,640 keV/$\mu$m) or Fe-ions (2,000 keV/$\mu$m). About 20% of all cells were judged to be non-hit, and about 10% cells survived radiation damage. About 70% cells died after dividing at least once (reproductive death) or without dividing (interphase death). RBE for reproductive (RBE[R]) and interphase (RBE[I]) death showed a similar LET dependence with maximum around 200 keV/$\mu$m. In this LET region, at 30% survival level, about 10% non-survivors underwent interphase death. The corresponding value for very high-LET Fe-ions (2,000 keV/$\mu$m) was not particularly high (~15%), whereas that for X-rays was less than 3%. However, reproductive death (67%) predominated over interphase death (33%) even in regard to rather severely damaged cells (1% survival level) after exposure to Fe-ions (2,000 keV/$\mu$m). These indicate that interphase death is a type of cell death characteristic for the cells exposed to high-LET radiation and is not caused by ‘cellular over kill effect’. Both NHF37 (non-hit fraction at 37% survival) and inactivation cross-section for reproductive death (\(\sigma[R]\)) began to increase when LET exceeded 100 keV/$\mu$m. The exclusion of non-hit fraction in the calculation of surviving fraction partially prevented the fall of RBE[R] when LET exceeded 200 keV/$\mu$m. On the other hand, the mean number of lethal damage per unit dose (NLD/Gy) showed the same LET-dependent pattern as RBE[R]. These suggest that the increase in non-hit fraction and RBE took place with an increasing LET is caused by enhanced clustering of ionization and DNA damage which lowers the energy efficiency for producing damage and RBE.

INTRODUCTION

Although accelerated heavy particles such as Fe-ions, which are abundant in the space, contribute only a few percent of an astronauts’ overall radiation dose during space flight,1 the effect of biological damage and health risk may be significant. It is well known that the cell killing effect of ionizing radiation depends on LET and that RBE reaches a maximum at LET around 200 keV/$\mu$m,2 and then decreases abruptly, further declining to less than 1.0 in regard to very high LET heavy-ions such as $^{40}$Ar or $^{56}$Fe (atomic mass \(\geq 40\); LET > 1,000 keV/$\mu$m),3,4 ‘Cellular over kill’ hypothesis proposed by Barendsen et al.5 has been common interpretation for the fall of RBE with very high-LET, i.e. that cells are inactivated by single-particle traversals which deposit more energy than actually required. This interpretation assumes that one single particle traversal suffices for cell inactivation. On the other hand, Kiefer and colleagues have suggested that saturation occurs not at the cellular but the molecular level (molecular over kill); low-energy heavy ions deposit their energy in very small tracks so that only a limited number of target molecules are hit, and even if they are all inactivated their number may be too small to lead to cell killing.

Situation different from low-LET radiation has been
pointed out in regard to very high-LET radiation by Goodhead et al.\(^9\); an individual cell which survives irradiation with very high-LET ions may either have received no lethal lesions despite having been intersected by one or more tracks or it may have been missed entirely by the tracks. For instance, the fraction of CHO cells whose nuclei are not hit by 4 Gy of \(^{56}\)Fe-ions (2,000 keV/\(\mu\)m) has been demonstrated to be \(\sim 20\%\) by Mehnati et al.\(^9\) based on long term time-lapse observation of irradiated individual cells, whereas the surviving fraction was \(\sim 30\%\). In the present study, the reason for the fall of RBE in high-LET region was investigated by evaluating the fraction of non-hit cell and interphase death.

**MATERIALS AND METHODS**

**Cell culture**

A Chinese hamster ovary cell line (CHO-K1) provided by the RIKEN Cell Bank, Japan was used in this study. Cells were grown in monolayer in Ham’s F12 medium supplemented with 10% fetal calf serum at 37\(^{\circ}\)C in CO\(_2\) incubator. Mean cell-cycle time of CHO cells was \(\sim 12\) h.

**Irradiation**

Exponentially growing asynchronous CHO cells were exposed to monoenergetic ion-beams (dose rate, \(\sim 5\text{ Gy/min}\)) of appropriate LETs, which were adjusted with adequate energy degrader, by employing heavy-ion accelerators at RIKEN (RRC: RIKEN Ring Cyclotron), National Institute of Radiological Sciences (NIRS) (HIMAC: Heavy Ion Medical Accelerator in Chiba) and Japan Atomic Energy Research Institute (JAERI) (TIARA: Takasaki Ion Accelerators for Advanced Radiation Application). We used \(^{12}\)C-ions of HIMAC and RRC (135 MeV/nucleon: 20, 60, 82, 99 keV/\(\mu\)m), \(^{20}\)Ne-ions (135 MeV/nucleon: 102, 120, 171, 228, 341 keV/\(\mu\)m), \(^{40}\)Ar-ions (95 MeV/nucleon: 1,640 keV/\(\mu\)m) and \(^{56}\)Fe-ions (90 MeV/nucleon: 780, 1,200, 2,000 keV/\(\mu\)m) of RRC. \(^{29}\)Ar-ions of TIARA (11.5 MeV/nucleon: \(\sim 1,800\) keV/\(\mu\)m) and \(^{56}\)Fe-ions of HIMAC (200 MeV/nucleon: \(\sim 500\) keV/\(\mu\)m) were also used in the preliminary experiments. Ion-beams were delivered to the cells grown in Nunc T25 plastic flasks by RRC and HIMAC, but to those grown on CR-39 plastics within plastic dishes in the case of TIARA. Cells were also irradiated with 200 kVp X-rays (Shimazu Pantak X-ray generator; dose rate, \(\sim 2\) Gy/min).

**Time-lapse photography**

Non-hit cell fraction for reproductive death was determined by means of time-lapse observations of irradiated cells. Nunc T25 flask with ventilation ports in which cells were grown in monolayer was mounted on the stage of a phase-contrast microscope. Under continuous supply of 5% CO\(_2\)-95% air through these ports at 37\(^{\circ}\)C, photographs were taken every 30 minutes with a 35 mm motor-driven camera. Since the exposures were of the same microscopic field during preirradiation (\(\sim 20\) h) and postirradiation period (70–100 h), the cell stage of asynchronously growing cells at irradiation could be estimated by elapsed time after cell division. Pedigrees of individual cells exposed to Fe or Ar-ions were constructed by reviewing the films.

**Principal formulae and symbols used for obtaining the equations in the text**

The equations shown in the main text ([1]–[6]) and under the table ([7]–[9]) are derived from two of the following principal formulae ([a]–[j]).

<table>
<thead>
<tr>
<th>Principal formulae</th>
<th>F= 6.25×D/ LET</th>
<th>SF= (\exp (–D/ D_0))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF= (\exp (–\sigma[R]×F))</td>
<td>(\exp (–\sigma[I]×F))</td>
<td></td>
</tr>
<tr>
<td>(\exp (–\sigma[R]×F))</td>
<td>(\exp (–\sigma[I]×F))</td>
<td></td>
</tr>
<tr>
<td>NLD/ Gy[R]= (\sigma[I]×\sigma[R]×F/ D)</td>
<td>NLD/ Gy[I]= (\sigma[I]×\sigma[R]×F/ D)</td>
<td></td>
</tr>
<tr>
<td>(1–\exp (–\sigma[I]×F))</td>
<td>(1–\exp (–\sigma[I]×F))</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Survival curves of CHO cells exposed to X-rays and accelerated charged particles (C, Ne, Fe) of different LETs. Cell survival was determined by using the colony-forming ability as an indicator. Surviving fraction of X-irradiated cells was indicated as mean value ± SD. Exponential survival curves of accelerated charged particles and an exponential part of X-ray survival curve were drawn automatically using KaleidaGraph software which was based on least square regression analysis (The survival curves for X-rays and Fe-ions were reproduced from the original figure in the reference No. 9 with permission of Fukuoka Medical Society).
Exploration of Over Kill Effect of Heavy Ions  

**Symbols**

- $D$: dose (Gy)
- $D_0$: mean lethal (37% survival) dose
- $F$: particle fluence (particle/μm$^2$)
- $F_0$: mean lethal particle fluence
- $F_{63}$: particle fluence for induction of 63% interphase death
- $SF$: surviving fraction
- LET: linear energy transfer (keV/μm)
- $\sigma$: inactivation cross-section
- $\sigma[R]$: inactivation cross-section for reproductive death (μm$^2$)
- $\sigma[I]$: inactivation cross-section for interphase death
- $\sigma[R]$: target area for reproductive death (μm$^2$)
- $\sigma[I]$: target area for interphase death
- $NHF[R]$: non-hit fraction for reproductive death
- $NHF[I]$: non-hit fraction for interphase death
- $s_T[R]$: target area for reproductive death (μm$^2$)
- $s_T[I]$: target area for interphase death
- $NLD/Gy[R]$: mean number of lethal damage per unit dose for reproductive death
- $NLD/Gy[I]$: mean number of lethal damage per unit dose for interphase death

**Table 1.** LET-dependent changes in RBE for cell killing ($RBE[R]$: reproductive death, $RBE[I]$: interphase death) and other parameters in CHO cells exposed to various ions accelerated by HIMAC (C-ions) and RRC (C, Ne, Ar and Fe-ions).

<table>
<thead>
<tr>
<th>Ions</th>
<th>LET (keV/μm)</th>
<th>$D_0$ (Gy)</th>
<th>$\sigma[R]$; $\sigma[I]$ (μm$^2$)</th>
<th>$NHF_{37}*$ (%)</th>
<th>$RBE[R]$ ($RBE[R]=RBE[I]$)</th>
<th>NLD/Gy[R]$^a$; NLD/Gy[I]$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C$</td>
<td>20</td>
<td>1.5</td>
<td>2.1; 0.11</td>
<td>–</td>
<td>1.3; 1.2</td>
<td>79; 0.34</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.2</td>
<td>7.9; 0.35</td>
<td>–</td>
<td>1.6; 1.3</td>
<td>99; 0.36</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>1</td>
<td>13; 0.52</td>
<td>–</td>
<td>1.9; 1.8</td>
<td>120; 0.40</td>
</tr>
<tr>
<td></td>
<td>99</td>
<td>0.97</td>
<td>16; 0.74</td>
<td>–</td>
<td>2.0; 2.0</td>
<td>125; 0.47</td>
</tr>
<tr>
<td></td>
<td>102</td>
<td>0.91</td>
<td>18; 0.96</td>
<td>0.12</td>
<td>2.1; 2.0</td>
<td>130; 0.59</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.9</td>
<td>21; 1.2</td>
<td>0.17</td>
<td>2.1; 2.4</td>
<td>131; 0.63</td>
</tr>
<tr>
<td>$Ne$</td>
<td>171</td>
<td>0.76</td>
<td>36; 1.8</td>
<td>3.6</td>
<td>2.5 (2.6); 2.5</td>
<td>160; 0.66</td>
</tr>
<tr>
<td></td>
<td>228</td>
<td>0.81</td>
<td>45; 2.7</td>
<td>7</td>
<td>2.4 (2.7); 3.3</td>
<td>150; 0.74</td>
</tr>
<tr>
<td></td>
<td>341</td>
<td>0.97</td>
<td>57; 3.5</td>
<td>12</td>
<td>2.0 (2.6); 2.6</td>
<td>125; 0.64</td>
</tr>
<tr>
<td>$Ar$</td>
<td>1,640</td>
<td>3.3</td>
<td>80; –</td>
<td>23</td>
<td>0.58 (1.0); –</td>
<td>37; –</td>
</tr>
<tr>
<td></td>
<td>780</td>
<td>2.1</td>
<td>59; 5.3</td>
<td>13</td>
<td>0.9 (1.1); 1.5</td>
<td>57; 0.42</td>
</tr>
<tr>
<td>$Fe$</td>
<td>1,200</td>
<td>2.6</td>
<td>74; 6.7</td>
<td>20</td>
<td>0.73 (1.1); 1.2</td>
<td>47; 0.35</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>3.4</td>
<td>97; 8.4</td>
<td>29</td>
<td>0.56 (1.3); 1.0</td>
<td>36; 0.26</td>
</tr>
</tbody>
</table>

*Calculated according to the equation [3]; $^a$RBE[R] corrected by excluding non-hit cells; $^b$NLD/Gy[R]= $\sigma[R]$$\times$$\sigma[R]$$\times$6.25/LET [7]; $^c$NLD/Gy[I]= $\sigma[I]$$\times$$\sigma[I]$$\times$6.25/LET [8]. Parameters of X-ray survival curve: $D_0$=1.9 Gy, $D_q$=1.9 Gy, n=2.7.

**Table 2.** Estimation of target area for cell killing in CHO cells exposed to high-LET heavy ions.

<table>
<thead>
<tr>
<th>Ions</th>
<th>Reproductive Death</th>
<th>Interphase Death</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Ar$</td>
<td>$Fe$</td>
</tr>
<tr>
<td>LET (keV/μm)</td>
<td>1,640</td>
<td>2,000</td>
</tr>
<tr>
<td>Dose (Gy)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SF</td>
<td>0.3</td>
<td>0.31</td>
</tr>
<tr>
<td>$NHF[R]^*$</td>
<td>0.18</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(22 / 122)**</td>
<td>(29 / 142)**</td>
</tr>
<tr>
<td>$\sigma[R]$ (μm$^2$)</td>
<td>80</td>
<td>97</td>
</tr>
<tr>
<td>$\sigma[R]$(μm$^2$)</td>
<td>(mean)</td>
<td>(120)</td>
</tr>
</tbody>
</table>

*Calculated assuming $\sigma[I]= 10$ μm$^2$ (NHF[I]= exp $[-\sigma[I]$$\times$6.25×D]/LET)[9].

FID: fraction of cells undergoing interphase death

RESULTS

Estimation of non-hit fraction and cell target area for reproductive death

Survival curves of CHO cells exposed to X-rays and accelerated charged particles (C, Ne, Ar, Fe) of various LETs were made by using the colony-forming ability as an indicator of cell survival. As shown in Fig. 1 the survival decreased exponentially with the dose of accelerated charged particles such as C-ions higher than 20 keV/Å, whereas X-ray survival curve accompanied with the shoulder region (Dq=1.9 Gy) fitted well to a multi-targets and single hit model. Since the D0 values (2.1, 2.6, 3.4) for very high LET Fe-ions (780, 1,200, 2000 keV/Å) are larger than that for X-rays (1.9 Gy), X-ray and these Fe-ion survival curves intercross at a certain dose and survival level. A survival curve for Ar-ions (1,640 keV/μm) (not shown in Fig. 1) was similar to that for Fe-ions (2,000 keV/μm). Since RBE for reproductive death (the loss of colony-forming ability) (RBE[R]) was determined from D0 ratio in the present study, it was less than unity (1.0) with regard to these very high-LET heavy-ions. Inactivation cross-section for reproductive death (σ[R]) listed in Table 1 was calculated from the following equation.

\[
\sigma[R] = 0.16 \times \text{LET} / D_0
\]  

[1]

In the LET region lower than 100 keV/μm, \(\ln (\text{NHF}[R]) / \ln (\text{SF})\) is much higher than unity (1.0) but decreases and approaches to 1.0 with the increase in LET. The following relations are expected to hold for an extremely high-LET ion; \(\sigma_T[R] = \sigma[R]\) and NHF[R] = SF. NHF[R] = SF implies that all hit cells die and only non-hit cells can survive.

The target area was calculated by determining NHF[R] with time-lapse observations of irradiated cells for 6–7 generations after irradiation. Irradiated cells are divided into survivor and non-survivor groups. Survivor group consists of non-hit cells and the cells surviving irradiation by the repair of radiation damage during irradiated generation (LS negative survivor) or removal of remaining lethal damage.

Cell nucleus has been considered to be the target responsible for radiation-induced cellular effect such as cell death and division delay. However, target area may differ depending on type of effect. The target area for reproductive death (\(\sigma_T[R]\)) is related to \(\sigma[R]\) by the following equation.

\[
\sigma_T[R]\text{[μm}^2]\quad = \quad (\ln (\text{NHF}[R]) / \ln (\text{SF}) \times \sigma[R])\text{[μm}^2]\]  

[2]

In Fig. 2, Identification of reproductive death, LS positive survivor and non-hit cell by means of long-term time-lapse observations of CHO cells exposed to 4 Gy Fe-ions (LET: 2,000 keV/μm). \(^a\)Time after division (h) at irradiation; \(^b\)Time interval (h) from irradiation to first division; \(^c\)Cell-cycle time prolonged by delayed division delay (delay: 22–12 [mean cell-cycle time of unirradiated cells] = 10 h); \(^d\), delayed division delay.

In Fig. 3, Dose-response curves for induction of interphase death in CHO cells, determined by means of time-lapse observation of 100–150 cells for each data point (The curves were reproduced from the original figure in the reference No. 12 with permission of Radiation Research Society).
through postirradiation divisions (lethal sectoring)\textsuperscript{10,11} (LS positive survivor). Non-survivor group corresponds to the cells undergoing reproductive or interphase death. Outline of original pedigrees in our previous study\textsuperscript{9} is illustrated as an example of reproductive death (A), LS positive survivor (B) and non-hit cell (C) in Fig. 2. The cell was judged to be non-hit, if it showed no radiation-induced division delay at irradiated generation, no prolongation of cell-cycle time caused by remaining radiation damage during postirradiation several generations and no delayed death in its clonogenic progeny because of radiation-induced genomic instability.

Results are shown in the left column of Table 2. Non-hit fraction was estimated to be ~20% at ~30% survival, indicating that more than half (60–65%) of survivors were non-hit cells. The target area was calculated according to the equation \[2\] to be 130 and 113 m\(^2\) for Fe and Ar-ions, respectively. Mean value 120 m\(^2\) was adopted as the target area for reproductive death (\(s_T[R]\) of CHO cells).

Percent non-hit fraction at 37% survival (NHF\textsubscript{37}) listed in Table 1 was calculated in regard to the cells exposed to accelerated charged particles of different LETs according to the following equation.

\[
\text{NHF}_{37} = 37 \times \exp\left(-\frac{\sigma_R(R)}{\sigma(R)} - 1\right)
\]

The value obtained was about 30% for the highest-LET Fe-ions (2,000 keV/\(\mu m\)) and less than 0.2% in the LET region lower than 120 keV/\(\mu m\).

Estimation of non-hit fraction and the target area for interphase death

Radiation-induced cell death is categorized into reproductive and interphase death. We previously reported that interphase death was predominantly induced by high-LET ionizing radiation at irradiated generation and that this type of death was characterized by cell-cycle arrest with the accompanying giant cell formation and apoptosis.\textsuperscript{11,12} Fraction of the cells undergoing interphase death increased linearly with dose after exposure to high-LET charged particles such as Ne-ions, whereas it began to increase when the dose exceeded threshold of ~10 Gy for X-rays, as shown in Fig. 3.

RBE[I] measured as the ratio of dose required to induce 50% fraction of interphase death (D\textsubscript{50}[X-rays]/ D\textsubscript{50}[Ne-ions]) was about 3 with regard to 228 keV/\(\mu m\) Ne-ions. Inactivation cross-section for interphase death (\(\sigma[I]\)) was calculated for accelerated charged particles of different LETs according to the following equation and is listed in Table 1.

\[
\sigma[I] (\mu m^2) = \ln\left(\text{NHF}[I]\right) / \ln\left(1 - |\text{FID}|\right) \times \sigma[I] (\mu m^2)
\]

As described above, the cell target area for reproductive death (\(s_T[R]\)) was estimated to be ~120 \(\mu m^2\) (mean value) (the left column of Table 2), indicating \(s_T[R] = 1.2 \times \sigma[R]\) for Fe-ions (2,000 keV/\(\mu m\)). If the same relation holds for interphase death, \(s_T[I]\) will be 10 (1.2 \times 8.4) \(\mu m^2\). NHF[I] for 9.3 Gy of Ne-ions (228 keV/\(\mu m\)) and 27 Gy of Fe-ions (2,000 keV/\(\mu m\)) were calculated to be 0.08 and 0.43, respectively (the right column of Table 2).

Correlation between various parameters and the contribution of non-hit cell fraction to the decrease in RBE

LET-dependent changes in RBE[R], RBE[RC] (RBE[R] corrected by excluding non-hit fraction) and RBE[I] and relative values of NHF37, \(\sigma[R]\) and NLD/Gy[R] are shown in Fig. 4(a) and 4(b), respectively. Relative values were used in order to show correlation between these parameters. The fig-

![Fig. 4. (a) LET-dependent changes in RBE[R], RBE[RC] (RBE[R] corrected by excluding non-hit fraction) and RBE[I]. (b) Relative values (% maximum) of NHF37, \(\sigma[R]\), NLD/Gy[R] and RBE[I].](http://jrr.jstage.jst.go.jp)
The probability was calculated according to the equation \[ p \text{ for interphase death} = \frac{[1-\exp (\sigma[I]/\sigma[R] \times \ln [SF])] }{(1-SF)} \] at the LET lower than 100 keV/\mu m but increases thereafter with an increasing LET; \( \sigma[R] \) increases with LET in a manner similar to NHF37 and is expected to reach the plateau corresponding to \( \sigma[R] \) at LET higher than 3,000 keV/\mu m; (3) RBE vs LET curves both for reproductive and interphase death show a similar pattern; (4) RBE[R] begins to decrease in the LET exceeding 200 keV/\mu m where NHF37 reaches about 10%; (5) However, the exclusion of non-hit fraction in the calculation of surviving fraction partially prevented the decrease in RBE[RC] in the LET higher than 200 keV/\mu m; (6) Correlation between RBE[R] and NLD/ Gy[R] is perfect. These facts suggest that an increase in the degree of clustering of initial physical damage within target area with an increasing LET is the cause for the appearance of non-hit fraction and the decrease in the mean number of lethal damage per unit dose, resulting in the decrease of RBE for cell killing, although the killing effect of a single heavy-particle increases further with the accompanying increase in inactivation cross-section.

**Interphase death and over kill effect**

Since interphase death could not be induced by low-LET X-rays unless the dose delivered to the cells was larger than 10 Gy (Fig. 3), it seemed as if interphase death occurred exclusively in severely damaged cells. The following indicates that this might be not the case.

Probability for non-surviving cells to undergo interphase death is given by the following equation.

\[
p = \frac{1 - \exp \left( \frac{\sigma[I]}{\sigma[R]} \times \ln [SF] \right)}{1 - SF}
\]

Values at 1, 10 and 30% survival level are shown in Fig. 5 for the cells exposed to X-rays, C-ions (60 keV/\mu m), Ne-ions (228 keV/\mu m) and Fe-ions (2,000 keV/\mu m). The figure demonstrates that interphase death occurs in significant amount even at relatively high survival level with regard to high-LET radiation, and that the probability is higher in the LET at over kill (Fe-ions) than RBE peak region (Ne-ions) but the difference is not particular. However, reproductive death (67%) predominates over interphase death (33%) even at low survival level (1%) after exposure to Fe-ions (2,000 keV/\mu m). Interphase death may be a type of cell death that is characteristic for high-LET ionizing radiation rather than a manifestation of over kill effect at cellular level.

Finally it should be pointed out that judgment based on the loss of colony-forming ability can not discriminate reproductive and interphase death, although this assay has been usually used as an indicator of radiation-induced reproductive death.

**DISCUSSION**

Since the effect of penumbra in the track-structure of heavy-ions greater than \( ^{40} \text{Ar} \) on cell killing could not be ignored and hence the radiation-sensitive cellular target area for reproductive death (\( \sigma[R] \)) might be larger than actual geometric area of cell nucleus, the target area was determined empirically from non-hit cell fraction (NHF[R]) which was determined by means of time-lapse observations of irradiated cells. Calculated mean value of CHO cells (120 \( \mu \text{m}^2 \)) was similar to but slightly larger than geometric nuclear area of CHO-tsH1 cells (113 \( \mu \text{m}^2 \)). This suggests that non-hit fraction can be roughly estimated from the principal formula [d] in which \( \sigma[I] \) is replaced by geometric nuclear area.

The cell target area for interphase death (\( \sigma[I] \)) was estimated to be \( \approx 10 \mu \text{m}^2 \) and less than one tenth of that for reproductive death (120 \( \mu \text{m}^2 \)). This implies that the probability for a single high-LET heavy-ion to induce interphase death is much lower (\( \approx 1/12 \)) than that to induce reproductive death. Although centrosome seems to be one of the candidates as the target for interphase death because of its important role in cell division, its size (\( \approx 200 \text{ nm} \) in diameter) is too small to be the target. Alternatively we assume that more than 10 hits to nuclear area are required to generate more than one non-rejoining chromosome breaks which arrest the cells at checkpoints during the cell-cycle and commit them to undergo apoptosis. Evidence that the DNA content of the cells arrested before mitosis for long duration (\( \approx 20 \text{ h} \)) post irradiation was significantly larger than that of G2-phase cells (data not shown) suggests the cells’ entry to S from G2-phase with no accompanying cell division, leading to giant cell formation and interphase death.

LET-dependent pattern of RBE[R] and RBE[I] was similar in that the value began to decrease when LET exceeded...
200–300 keV/μm, although the former further decreased to less than 1.0 in very high LET region (780–2,000 keV/μm). However, the exclusion of non-hit fraction in the calculation of surviving fraction partially prevented such decrease in RBE[R] (Fig. 4[a]). Perfect correlation between NLD/Gy[R] and RBE[R] over entire LET examined (20–2,000 keV/μm) (Fig. 4[b]) indicates that LET-dependent change in RBE[R] correlates with change in the efficiency for producing radiation damage. On the other hand, NHF37 and σ[R] increased in a similar LET-dependent manner and seemed to reach the first plateau at LET ~300 keV/μm (Fig. 4[b]) but to increase again from LET ~800 keV/μm. The value of σ[R] is 97 μm² at LET 2,000 keV/μm (Table 1) and will reach the second plateau corresponding to σ[R] (120 μm²) at LET higher than 3,000 keV/μm. Further detailed study should be done in order to confirm this LET dependence. These suggest that the increase in non-hit fraction and inactivation cross-section (σ[R]) with an increasing LET is caused by enhanced clustering of ionization and DNA damage which lowers the energy efficiency and RBE.

In Fig. 6 the fates of the cells exposed to 4 Gy Fe-ions (2,000 keV/μm; 30% survival dose) are illustrated. About eighty percent cells were hit by Fe-ions and ~20% cells were non-hit. About ten percent hit cells could survive as they removed lethal damage through postirradiation 1st–4th divisions (lethal sectoring) (see Fig. 2B). Damage removed as lethal sector was manifested in the form of cell abnormality and death in the progeny. Clonogenic progenitor (clonogen) appeared during the process of lethal sectoring. If non-lethal damage, which is difficult to remove through cell division, is remaining in the clonogens, genomic instability will be induced in their progeny.

![Diagram](https://example.com/diagram.png)

**Fig. 6.** Fates of CHO cells exposed to 4 Gy Fe-ions (2,000 keV/μm). *Offspring without reproductive integrity; †Surviving fraction corrected by excluding non-hit fraction (20%) from survivors and irradiated cells.

A possible candidate of non-lethal damage is potentially unstable chromosome region (PUCR) which may be created at the DNA double-strand breaks (DSB)-rejoining sites through repair/ misrepair, as suggested by Suzuki. Heavy-ions are assumed to generate clustered PUCR via clustered DNA damage. Delayed DNA DSB at PUCR sites with the accompanying reactivation of DNA damage responding gene products (p53, NBS1, CHK2) might trigger delayed division delay (DDD). One of the authors (H. S.) has recently reported for the first time about alpha-particle induced DDD in HeLa cells and high RBE (9–14) of alpha-particles for the induction of delayed cell death in the clonogenic progeny. An example of Fe-ion induced DDD is shown in Fig. 2B. Delayed division delay because of delayed chromosome breakage will result in delayed cell death.

Presence of non-hit cells among hit cells is one of the features characteristic for exposure to very high-LET heavy-ions. Bystander effect may occur in non-hit cell. This effect will be ascertained if heavy-ion’s hit or non-hit to individual cells can be judged accurately e.g. by using CR-39 plastics. Furthermore, study on non-lethal effect of heavy-ions will be important for the assessment of radiation environmental health hazard to astronaut in the space, because high-LET charged particles have a higher probability to induce non-lethal than lethal damage.

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**REFERENCES**

7. Kiefer, J (1985) Cellular and subcellular effects of very heavy...

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