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Change of cell growth and mitochondrial membrane polarization in the progeny of cells surviving low-dose high-LET irradiation from Ra-226

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Abstract

In order to test the delayed effect of radiation on the progeny of irradiated survivors, the human keratinocyte cell line HaCaT and the fish common bluegill embryonic cell line CHSE/F were exposed to low-dose high-LET α-radiation from Ra-226 or γ-rays. The clonogenic survival fraction, mitochondrial membrane polarization (MMP) and reproductive ability of the descendants of the surviving cells were measured. For progeny of irradiated HaCaT survivors, no delayed cell death occurred. On the contrary, progeny at about 47 cell doublings after Ra-226 irradiation and progeny at about 14 cell doublings after γ-irradiation showed increased clonogenic survival. However the total cell number was reduced for progeny of Ra-226-treated cells up to about 47 cell doublings after irradiation and for progeny of γ-irradiated cells up to about 28 doublings after irradiation, which means low reproductive ability had appeared. In addition, α-radiation from Ra-226 had greater impact on the MMP of the HaCaT progeny than γ-rays. MMP of progeny of Ra-226-treated cells decreased at 5 cell doublings after irradiation and increased dose-dependently at 19 cell doublings after treatment, and then decreased dose-dependently at 47 cell doublings, while there was no significant effect on MMP in progeny of γ-irradiated cells. The progeny of Ra-226-irradiated CHSE/F survivors showed more serious damage than the offspring of γ-irradiated CHSE/F cells. Significant, dose-dependent delayed cell death occurred in progeny of surviving cells up to about 61 cell doublings after Ra-226 treatment, and the reproductive ability was also significantly reduced. But the MMP increased, which might be because of the increased removal of dead cells. For progeny of CHSE/F cells surviving γ-rays radiation, no significant change in clonogenic survival occurred, except for offspring of cells surviving low dose (0.1Gy
and 0.5Gy) irradiation, which had higher survival than control up to about 28 cell doublings after irradiation. But the number of cells which were the progeny of γ-irradiated survivors decreased dose-dependently up to about 28 cell doublings after γ-irradiation.

Keywords: lethal mutation, delayed cell death, mitochondrial membrane depolarization, low-dose, alpha radiation

1 Introduction

The long-term low-dose exposure of humans and other animals to naturally occurring α-emitting radium isotopes and their radioactive decay products has attracted increasing attention due to the increase of uranium mining and milling for nuclear power generation. The α-particles can cause severe biological damage because of the high linear energy transfer (LET) and long half-life. Reported adverse health effects of exposure to radium include various types of cancers in bone, lung and liver, leukemia, inhibited reproduction and reduced biomass in human and non-human species (Brugge and Buchner, 2012; Lourenço et al., 2012; Mothersill et al., 2013; Shi et al., 2017). Abnormal expression of apoptosis related genes and oxidative stress were also reported in fish embryo cells treated with environmentally relevant level of Ra-226 (Olsvik et al., 2012). The previous studies by our laboratory looking at the outcome of chronic low-dose exposure to Ra-226 radiation also revealed that decreased clonogenic survival occurred in irradiated HaCaT cells, and that the mitochondrial membrane of irradiated HaCaT and HCT116 p53+/+ cells depolarized and collapsed (Shi et al., 2016; Vo et al., 2017). We suggested that delayed lethal mutation or genomic instability may be involved in the damage response of cells exposed to long-term low-dose high-LET radiation.

Lethal mutation or the delayed expression of cell death in progeny of irradiated survivors is considered to be one manifestation of genomic instability (Mothersill and Seymour, 1997a). Genomic instability (Seymour et al., 1986), bystander effect (Nagazawa and Little, 1992, Mothersill and Seymour, 1997b) and adaptive response (Olivieri et al., 1984) are all considered to be non-targeted effects (NTE) of radiation. DNA in the cell nucleus
is believed to be the main target of ionizing radiation, but the discovery of NTE indicates that the direct DNA radiation damage theory is insufficient, especially after exposures to doses less than 1Gy or dose rates less than 0.1Gy/day (Prise et al., 2002). A high yield of lethal mutations in the descendants of γ-rays irradiated CHO-KI cells was first reported by Seymour et al. in 1986 (Seymour et al., 1986) and has since been shown in many other cell lines and systems (Mothersill et al., 1998; O’Reilly et al., 1994; Dowling et al., 2005). Although genomic instability was shown to occur after acute alpha radiation exposure (Kadhim et al. 1992), most of the delayed reproductive death research involved the use of low-LET radiation. Yields of delayed lethal mutations after high-LET irradiation, such as neutrons and α-particles were seldom reported (Mothersill et al., 2000), and there are even fewer studies of NTE using chronic high-LET irradiation.

In current study, the clonogenic survival, mitochondrial membrane polarization and reproductive ability were tested in the descendants of cells surviving chronic low-dose Ra-226 irradiation, and comparisons were made with low-LET γ-irradiation. The objective of this work is to see whether or not lethal mutations could be induced by chronic high-LET radiation from environmentally relevant levels of Ra-226 and to study the possible mechanisms, since there have been no previous studies of lethal mutation induction by chronic low-doses of high-LET radiation.

2 Materials and methods

2.1 Cell culture

A human epithelial cell line HaCaT and a fish embryonic cell line CHSE/F were used in the current study. The HaCaT cell line is an immortalized, nontransformed human keratinocyte cell line (Boukamp et al., 1988). The CHSE/F cell line was derived from common bluegill embryo. HaCaT and CHSE/F were initially obtained as generous gifts from Dr.Petro Boukamp (German Cancer Research Center, Heidelberg, Germany) and Dr. Niels Bols (University of Waterloo, Waterloo, ON, Canada). These two cell lines were previously used in our laboratory to study biological effects of acute and chronic γ-rays and α-particles irradiation (Shi et al., 2016a, 2016b; Vo et al., 2017a, 2017b).
HaCaT cells were cultured in complete growth medium contained RPMI1640 media, 10% FBS, 5ml of 100U/ml penicillin and 100μg/ml streptomycin sulphate, 5ml of 2mM L-glutamine, 25mM HEPES (Gibco Life Technologies Inc., Burlington, ON, Canada) and 1μg/ml hydrocortisone (Sigma-Aldrich, Oakville, ON, Canada) at 37 °C with 5% CO₂. CHSE/F cells were maintained in Leibovitz’s L-15 medium supplemented with 12% FBS, 100U/ml penicillin, 100μg/ml streptomycin sulphate, 2Mm L-glutamine and 25mM HEPES (Gibco Life Technologies Inc., Burlington, ON, Canada) at 20°C.

2.2 Irradiation

Direct α irradiation was performed by culturing cells in medium containing α-emitting radioisotope Ra-226. The stock Ra-226 was supplied as neutralized radium nitrate by Eckert and Ziegler (Valencia, CA, USA), and its activity concentration was 74Bq/μl. The stock 500Bq/ml Ra-226-medium was prepared in fresh RPMI 1640 or L-15 medium, the PH of it was measured with PH testing paper (Cole-Parmer, Montreal, QC, Canada) and was neutralized with NaOH solution (Sigma-Aldrich, Oakville, ON, Canada). Then it was filtered into storage tubes with a 0.22-μm Acrodisc syringe filter (VWR, Bridgeport, NJ, USA). The stock Ra-226-medium was diluted to make medium containing 200, 100, 10, 1 and 0.1Bq/ml Ra-226. At the beginning of the experiment, 500 of HaCaT cells were seeded into T25 culture flasks with 5ml of 0, 0.1, 1, 10, 100, 200 or 500Bq/ml Ra-226-medium and maintained at 37 °C with 5% CO₂ for 9 days. 600 of CHSE/F cells were seeded into and incubated in radioactive medium for 28 days at 21 °C without CO₂. Gamma irradiation was performed using the Taylor Source at McMaster University, which is a Caesium-137 source. Cells were seeded into flasks 6 hours prior to irradiation at room temperature (21 °C) with the Caesium-137 source which delivered a dose of 0.226Gy/min at the source-to-flask distance of 31cm. The doses of γ-radiation received by HaCaT cells or CHSE/F cells were 0.01, 0.1, 0.5, 1, 2 and 5Gy. After irradiation, cells were immediately returned to the incubator.

2.3 Clonogenic assay Technique
The clonogenic survival of cells treated with radioactive medium or $\gamma$-rays was assessed using clonogenic assay technique first described by Puck and Marcus (1956). Briefly, cells were detached and resuspended in medium, and the concentration of viable cells in the cell suspension was determined with a Z2 Coulter particle count and size analyzer (Beckman Coulter Electronics, Mississauga Ontario, Canada). Then appropriate numbers of cells were seeded into four flasks per dose point and were cultured in radioactive Ra-226-medium or control medium (with or without external exposure using the caesium source) for long enough to allow visible colony formation (For HaCaT cells this time was 9 days; for CHSE/F cells, 28 days were required). Then the three flasks for initial clonogenic survival estimation were stained with carbol fuchsin (VWR, Bridgeport, NJ, USA) to determine the survival fraction of irradiated cells ($P_0$), and the remaining flask was grown on to test the lethal mutation frequency in the population of surviving cells. For cells exposed to Ra-226-medium, the radioactive medium in the remaining flask was removed and the cells were washed out with 5ml of phosphate buffered saline (DPBS) (Gibco, Burlington, ON, Canada) for 5 times followed by a wash with complete cell culture medium. After this Ra-226 residues in the flasks were insignificant and couldn’t be detected with the detector which can detect alpha particles as well as beta particles and gamma rays (Radiation Measurement Systems, ON, Canada). Finally 5ml of fresh medium was added into the flask and the cells were returned to the incubator for culture. For cells irradiated with $\gamma$-rays, the medium in the remaining flasks was discarded and was replaced with 5ml fresh medium. Medium changes were given weekly until the cultures became 80%-90% confluent. Then clonogenic assay was performed again ($P_1$), and another set of flasks were set up, in which three were flasks stained for colony counting and one was used for further culture. But there was no further irradiation of these flasks. The procedure was repeated for several passages, lasting for about 70 doubling times for HaCaT cells and for CHSE/F (shown in Fig.1). At each passage, the number of cells per flask was determined, the plating efficiency relative to the sham-irradiated control was calculated and the mitochondrial membrane potential of cells was measured. The experiment was repeated three times.
2.4 Measurement of the mitochondrial membrane potential (MMP)

The mitochondrial membrane potential (MMP) was measured in the progeny of cells surviving α-irradiation from Ra-226 or γ-irradiation with the Mito Pt JC-1 mitochondrial permeability transition Kit (Cedarlane, Burlington, ON, Canada). For each experimental sample, two million cells detached from the culture flask were collected by 5-minute centrifugation at 1000rpm. The cell pellets were resuspended in 0.5ml of JC-1 working solution and were incubated for 15 minutes at 37 °C. Following incubation, the JC-1 stain was eliminated by 5-minute centrifugation at 1000rpm. The cell pellets were washed with DPBS once and then was resuspended in 1ml of DPBS. 100μl of the cell suspension was subsequently added into the well of a 96-well black wall plate to achieve 2×10^5 cells in each well and each sample was pipetted into 3 well replicates. Fluorescence spectroscopy was performed with the Tecan Infinite M200 Pro micro plate reader (Tecan, Switzerland) and the i-control sofware at the BioInterfaces Institute of McMaster University. The excitation was set to 488nm and dual emission wavelengths was set to 590nm for red fluorescence and 527 for green fluorescence. The ratio of red to green fluorescence, which was referred to as MMP ratio, was calculated to assess the mitochondrial membrane potential. Healthy cells have high MMP ratio because JC-1 accumulates within the mitochondria and more red-fluorescing J-aggregates exist. On the contrary, JC-1 distributes more evenly throughout the cytosol in apoptotic or otherwise stressed cells and more green-fluorescence monomers exist, resulting in a lower ratio of red/green fluorescence. The experiment was repeated at least three times independently.

2.5 Statistical Analysis

All data are expressed as means±SEM (the standard error of the mean). Various endpoints of progeny of irradiated HaCaT and CHSE/F survivors were analyzed by two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed using
Fisher’s least significant difference (LSD) test (SPSS22). A confidence interval of 95% and p<0.05 was selected to be statistically significant.

3 Results

3.1 Survival fractions of progeny of HaCaT cells surviving Ra-226 or γ-irradiation

Five-hundred HaCaT cells were seeded and cultured in 5 mL of medium containing 1, 10, 100, 200 and 500Bq/mL Ra-226 for 9 days. The cumulative doses that the cells received during this period are calculated as we described previously (Shi et al., 2016) and shown in Table 1. The treatment of low-dose alpha radiation did not have a large effect on the clonogenic survival of cells, except for two dose points, 0.0594mGy and 297mGy (Figure 2a). The survival of cells exposed to 0.0594mGy cumulative dose of alpha radiation was 109.2±2.7% (p<0.05), significantly higher than that of the control (100.0±1.5%). The clonogenic survival of cells irradiated by 500Bq/ml Ra-226 in medium for 9 days (297mGy accumulative dose) was significantly decreased to 92.3±1.6% (p<0.05).

The percentage survival of the progeny of cells which survived the Ra-226 irradiation for five passages (P1-P5, about 70 cell doublings) is shown in Figure 2b-2f. Delayed cell death only occurred in progeny of the highest-dose group at P1 (14 cell doublings), giving a survival fraction of only 83.5±2.8% (p<0.05), compared with 100%±2.6% for the control (Figure 2b). On the contrary, the clonogenic survival was significantly increased at the P4 passage for progeny of each irradiated group when compared to the progeny of control, and in general the increase was dose-dependent (Figure 2e).

Table I. Dose of irradiation received by HaCaT cells from α-particles emitted from Ra-226 in medium

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HaCaT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra-226 concentration in medium (Bq/ml)</td>
<td>0 0.1 1 10 100 200 500</td>
</tr>
<tr>
<td>Dose rate (mGy/d)</td>
<td>0 0.0066 0.066 0.66 6.6 13.2 33</td>
</tr>
<tr>
<td>days of culture in medium</td>
<td>9 9 9 9 9 9 9</td>
</tr>
<tr>
<td>Total dose of radiation (mGy)</td>
<td>0 0.0594 0.594 5.94 59.4 118.8 297</td>
</tr>
</tbody>
</table>
The initial survival curve of HaCaT cells irradiated to 0-5 Gy γ-rays with Cs-137 is shown in Figure 3a. There is a dose-dependent decrease in the initial cell survival. Delayed cell death did not occur in the progeny of cells surviving irradiation with γ-rays, while increased survival appeared in P1 of 0.01, 0.05, 0.1 and 2 Gy irradiated survivors, as well as P2 of 0.5 Gy and 2 Gy irradiated survivors (Figure 3b-f).

3.2 Change of mitochondrial membrane potential (MMP) in progeny of HaCaT cells surviving Ra-226 or γ-irradiation

When the HaCaT cells were almost confluent and ready for subculture, the mitochondrial membrane potential of cells was measured. Mitochondrial membrane depolarization occurred in HaCaT cells when they had been cultured in radioactive medium for 9 days and in non-radioactive medium for 5 further days (Figure 4a). At this time, the MMP ratios of descendent of cells exposed to 0.0594, 0.594, 5.94, 59.4 and 118.8 mGy alpha-radiation of Ra-226 were 0.69±0.03, 0.77±0.05, 0.70±0.01, 0.76±0.02 and 0.78±0.03 (mean±SD, n=9, p<0.05). At the 2nd subculture, the mitochondrial membrane of progeny of irradiated survivors was not depolarised but rather the MMP ratios were higher than the control, and were respectively 26%, 27%, 42%, 32%, 46% and 54% higher than control (Figure 4b). In the P3 progeny the depolarization of mitochondrial membrane occurred again, and depolarisation was even greater in the P4 survivors. In the P3 progeny of cells survived 0.594 mGy and 5.94 mGy radiation, the MMP was only 0.7±0.01 and 0.71±0.08, significantly lower than the control of 1±0.08 (n=9, p<0.05). The MMP ratios of P4 progeny were 0.82±0.02, 0.71±0.04, 0.62±0.03, 0.43±0.04 and 0.57±0.05 for 0.594, 5.94, 59.4, 118.8 and 297 mGy group, compared with 1±0.07 of the control (n=9, p<0.05). By the end of the experiment (P5), MMP ratios of the irradiated cells had recovered and were comparable to that of the control.
In the progeny of HaCaT cells surviving γ-irradiation, the change of MMP was not so marked. The MMP ratios only changed in progeny of cells at several dose points (Figure 5). For the first two progeny measurements (P1 and P2), no mitochondrial membrane depolarization occurred, on the contrary, progeny of relatively high-dose irradiated cells showed high MMP ratios. For example, MMP ratios of the P1 progeny of 0.5Gy and 5Gy γ-rays irradiated cells were 1.83±0.07 and 1.42±0.06, compared with the control of 1±0.04 (n=9, p<0.05); for P2 progeny, cells in 0.5, 2 and 5Gy groups had higher MMP ratios of 1.62±0.03, 1.23±0.09, 1.18±0.02 (n=9, p<0.05). At the P3 progeny, the high MMP ratios returned to the normal level, and were comparable to that of the control. At the P4 progeny, the MMP ratios of progeny of cells irradiated with 0.05, 0.1 and 5Gy γ-rays were only 0.71±0.09, 0.59±0.07, 0.57±0.04 respectively, compared with 1±0.08 of control (n=9, p<0.05). For the P5 progeny, the MMP ratios of cells in 0.1 and 5Gy groups were 0.59±0.05 and 0.73±0.05, and the control is 1±0.04 (n=9, p<0.05).

3.3 Delayed reproductive ability in progeny of HaCaT cells surviving treatment with Ra-226 or γ-irradiation

The reproductive abilities of HaCaT cells, which were the progeny of cells surviving different doses of radiation from Ra-226 in medium, were evaluated by counting the total cell number in every passage and determining the number of cell doubling in the time between plating and counting (shown in Figure 6). At the time of the first subculture, the numbers of cells which were the progeny of cells surviving 0.0594 to 5.94mGy α-irradiation, were 45±13%, 39±9% and 34±12% higher than the control (n=9, p<0.05). After the second passage, the progeny of cells surviving α-irradiation began to show the decreased reproductive ability compared to the control. At that time, the numbers of cells which were the offspring of 0.594, 5.94, 59.4 and 297 mGy Ra-226 irradiated cells were 0.54±0.08, 0.67±0.05, 0.73±0.1 and 0.70±0.09 respectively (n=9, p<0.05), compared with
1±0.06 of the offspring of control. The cell numbers of the P4 progeny of all Ra-226 treated groups were still lower than control, and were only 0.83±0.02, 0.75±0.07, 0.78±0.09, 0.60±0.04, 0.53±0.02 and 0.63±0.04 of that of control (n=9, p<0.05). At the end of the experiment (P5), the reproductive ability of cells had recovered to the level of control, and the relative cell number of offspring of 297mGy Ra-226 irradiated cells were even 23±5% higher than that in control.

For progeny of HaCaT cells surviving γ-rays radiation, the reproductive ability was also influenced (Figure 7). For the P2 progeny, the relative cell number in 0.1Gy γ-rays irradiated group were 1.26±0.04, while the relative cell number in 0.5, 2 and 5Gy γ-rays irradiated group were only 0.52±0.05, 0.53±0.02 and 0.14±0.01, compared to the control of 1±0.01 (n=9, p<0.05) (Figure 7b). The increase of the relative cell number in the low dose group and the decrease in the relatively higher dose group were maintained for another passage (P3, Figure 7c). After that, the reproductive ability of cells which were the offspring of cells survived radiation began to recover. But until the 5th passage (P5), the relative number of progeny of cells survived 5Gy γ-rays was 0.71±0.04, still lower than control (Figure 7e).

3.4 Survival fractions of progeny of CHSE/F cells surviving Ra-226 treatment or γ-irradiation

600 CHSE/F cells were kept in 5 mL medium containing 0.1, 1, 10, 100, 200, 500Bq/mL Ra-226 for 28 days. The clonogenic flasks were stained and cells in the remaining flasks were rinsed and continued to be cultured in fresh normal medium for other 14 days. The dose of radiation that CHSE/F cells received from Ra-226 was summarized in Table 2.

Table II. Dose of irradiation received by HaCaT cells from α-particles emitted from Ra-226 in medium

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CHSE/F concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ra-226</td>
<td>0  0.1  1  10  100  200  500</td>
</tr>
<tr>
<td>medium (Bq/ml)</td>
<td>Dose rate (mGy/d)</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Days of being cultured in medium</td>
<td>28</td>
</tr>
<tr>
<td>Dose of radiation (mGy)</td>
<td>0</td>
</tr>
</tbody>
</table>

Low-dose Ra-226 irradiation in the original medium could affect the clonogenic survival of the CHSE/F cells which were not exposed directly but were the progeny of cells surviving this irradiation, and the effect lasted for approximately 61 cell doublings (Figure 8). For cells irradiated to 0.1848, 1.848, 18.48, 184.8, 369.6 and 924 mGy accumulated α-radiation from Ra-226 in medium for 28 days, the survival fraction was 68.58±2.5%, 71.07±4.4%, 75.42±2.4%, 54.93±2.1%, 31.46±2.16% and 0% (n=9, p<0.05). Meanwhile, the survival fraction for unirradiated cells was 100±1.63% (n=9). For the P1 progeny of the survived cells, the clonogenic survival was still lower than the progeny of the unirradiated cells, and was 72.34±3.88%, 78.47±2.98%, 81.44±4.46%, 71.67±1.24% and 46.29±1.97% respectively for the original doses from 0.1848 to 369.6mGy. By P2, the survival began to recover and only at two dose-points, did the reduction in survival reach significance. They were 0.1848mGy and 184.8mGy, and the survivals were 8.79% and 11.05% lower than control (n=9, p<0.05). For P3 and P4, the reduced clonogenic survival in the progeny of cells survived Ra-226 irradiation could still be seen, and was 82.05±1.3%, 84.12±2.63%, 72.12±3.23%, 76.38±2.6% and 88.67±6.23% for doses from 0.1848mGy to 369.6mGy for P3 (n=9, p<0.05), 83.98%±2.52, 74.62±2.53%, 87.99±2.98% and 90.24±2.09% for doses from 1.848mGy to 369.6mGy for P4 (n=9, p<0.05). At the P5, the clonogenic survival for the progeny of cells which survived the original irradiation was not significantly different from the controls except for two dose points, 1.848mGy and 18.48mGy. The survival was 90.61±2.72% and 89.59±3.08% respectively for these two dose points (n=9, p<0.05).
For CHSE/F cells irradiated with γ-rays, the experiment was only carried to the second passage progeny (P2) because of the very long time needed to grow the cells to confluence. The survival curve of the original cells irradiated with γ-rays and their progenies were shown in Figure 9. For the cells irradiated with γ-rays, their survival showed a dose-dependent relationship and decreased as the increase of the dose of radiation. Unexpectedly, the reduction of clonogenic survival did not occur in the progeny of the survivors of the γ-irradiation. On the contrary, the survival of P1 cells, which were the offspring of 0.1Gy and 0.5Gy irradiated cells, were higher than that of the control (100±3.84%) and were 128.47±4.72% and 118.17±4.38% (n=9, p<0.05). Increased clonogenic survival happened in the same two groups at P2, and were 155.64±2.77% and 139.79±5.22% (n=9, p<0.05).

3.5 Change of mitochondrial membrane potential in progeny of CHSE/F cells surviving Ra-226 treatment or γ-irradiation

The change of mitochondrial membrane potential in the progeny of CHSE/F cells surviving the Ra-226 irradiation is shown in Figure 10. Generally speaking, in the five passages after the irradiation, the MMP ratios were higher for the progeny of cells surviving the radiation compared to the offspring of the unirradiated cells. This means that the polarization of the mitochondrial membrane was greater than in the control. At P1 and P2, the MMP ratios of cells increased as the increase of the dose of radiation that received by their progenitors except for the highest dose point at P1. At the beginning of P3, the increased MMP ratio only occurred at the highest two dose points, 184.8mGy and 369.6mGy, and the value was 1.37±0.03 and 1.54±0.08, when the value for the control was 1±0.01 (n=9, p<0.05). For P4 and P5, almost all the cells from the survivors of the original exposure to radiation showed significantly increased MMP ratio but there is no dose-dependent relationship.
For the progeny of CHSE/F cells surviving the γ-rays exposure, increased MMP ratio was also seen, such as at the P1 and P2 progeny, and a dose-dependent relationship was seen at the P2 progeny. For the P1 progeny, the MMP ratio for dose-points 0.05, 0.5 and 2Gy was 1.24±0.05, 1.31±0.07 and 1.36±0.09 respectively (n=9, p<0.05). For the P2 progeny of cells surviving 0.05, 0.1, 0.5, 2 and 5Gy γ-irradiation, the MMP ratios were 1.26±0.07, 1.45±0.07, 1.76±0.05, 1.78±0.14 and 1.66±0.05 (n=9, p<0.05). But at the P3 progeny, the increased MMP ratio disappeared and the ratio value decreased at two groups whose progenitor survived 0.1 and 5Gy γ-irradiation, and were 0.80±0.01 and 0.76±0.05 respectively (n=9, p<0.05).

3.6 Delayed reproductive ability reduction in progeny of CHSE/F cells surviving Ra-226 treatment or γ-irradiation

The relative cell number of the progeny of irradiated groups are shown in Figure 12, and from the figure, we can see that the reproductive ability of cells was influenced by the Ra-226 irradiation of their progenitors. At the P1 progeny, the cell number decreased in all irradiated cells and was decreasing as the increase of the dose of radiation, the relative cell number was 0.54±0.03, 0.52±0.06, 0.44±0.02, 0.18±0.01 and 0.02±0.001 respectively (n=9, p<0.05). At the following passages (P2-P5), the dose-dependent relationship still occurred even though the increase of relative cell number only reached significance at the highest two doses.

For the progeny of CHSE/F cells surviving the γ-irradiation, the decreased reproduction ability also happened (in Figure 13). As to the relative cell number counted at the first time of subculture, the 0.05Gy group has a higher value, which was 1.23±0.03, and the 0.5, 2 and 5Gy groups has decreased value, which was 0.83±0.02, 0.87±0.03 and
0.54±0.01 respectively (n=9, p<0.05). At the P2 progeny, the relative cell numbers of cells which were the progeny of irradiated cells were all lower than the control, and were 0.70±0.04, 0.55±0.04, 0.52±0.05, 0.62±0.04, 0.55±0.03 and 0.33±0.05 respectively for 0.01 to 5 Gy of irradiation (n=9, p<0.05). At the time of the third subculture, the relative number of cells in the irradiated groups began to recover and only two groups (0.01 Gy and 0.05 Gy) have a lower value than the control. These values were 0.71±0.02 and 0.79±0.02 (n=9, p<0.05). The relative number of cells that were the progeny of 5 Gy irradiated cells was 1.24, higher than the control (n=9, p<0.05).

4 Discussion

**Chronic low-dose high-LET α-radiation from Ra-226 is effective in inducing lethal mutations**

This work indicates that chronic low-dose high-LET α-radiation from Ra-226 in the medium is more effective than γ-radiation in inducing lethal mutations (delayed reproductive death or genomic instability) in progeny of irradiated survivors. Survival of the progeny of irradiated survivors obtained using a clonogenic assay is usually used to assess lethal mutation frequency in the progeny of irradiated survivors since these were first described by Seymour et al., (1986). In current work, significantly reduced clonogenic survival was seen in progeny of CHSE/F cells surviving 28-day Ra-226 radiation and could last for about 61 cell doublings after the irradiation, for example, the decreased clonogenic survival in the descendants of CHSE/F cells surviving 0.18 Gy and 0.37 Gy cumulative dose of α-irradiation from Ra-226 (see Figure 8). However, decreased clonogenic survival did not occur in the progeny of γ-ray irradiated CHSE/F survivors. On the contrary, higher clonogenic survival was found in the P1 and P2 progeny of CHSE/F cells surviving 0.1 and 0.5 Gy γ radiation compared with progeny of unirradiated cells (Figure 9b, 9c). The results of relative cell number from one cell in progeny of Ra-226-exposed or γ-irradiated survivors also demonstrate that severer delayed reproductive
death could be induced by α-radiation from Ra-226 than by γ-ray, and the delayed death could last for longer time. Relative cell number of progeny of 0.18Gy and 0.37Gy accumulative α-radiated CHSE/F cell survivor was lower than the control up to 5 passages (about 61 cell doublings) after irradiation, while it showed the sign of recovery in the second passage progeny of γ-rays irradiated survivors (see Figure 12, Figure 13). HaCaT cells which survived Ra-226 treatment also showed much lower relative cell number until about 4 passages after the treatment than control cells, while the reduced relative cell number of γ-irradiated HaCaT survivors only lasted for about 3 passages after irradiation (Figure 6 and 7). More severe lethal mutation frequency was also reported in the descendants of HPV-G cells surviving 0.5Gy α-particle irradiation than 0.5Gy γ-irradiation, and the persistent reduction in colony-forming ability and greater expression of chromosomal instability were demonstrated in progeny of 0.5Gy α-irradiated survivors (Mothersill et al., 2000). The high yield of lethal mutations in progeny of α-irradiated survivors may because of the more genomic and chromosomal change induced by α-irradiation. A higher yield of sister chromatid exchanges than expected was reported in 60% of cells exposed to 0.05Gy α-particles even though only 3% of nuclei would have experienced a traversal (Deshpande et al., 1996).

**Decreased clonogenic survival did not occur in descendants of irradiated HaCaT survivors**

The progeny of HaCaT irradiated survivors seemed to have comparable or even higher clonogenic survival than the descendants of unirradiated cells. For example, the increased clonogenic survival was found in the P1 and P2 progeny of HaCaT cells surviving γ-radiation at some dose points (Figure 3b, c) and the P4 progeny of HaCaT cells surviving 9-day α-irradiation at all doses (Figure 2e). The reason that low dose of α-radiation didn’t induce lethal mutation in HaCaT cells might be that this low level of radiation didn’t cause adequate cell death in the directly irradiated cells, so the damage didn’t exceed the threshold to trigger the delayed toxicity in HaCaT cells. A threshold of initial toxicity has been reported to be needed for the induction of delayed cell death, and was found to be about 60% (Mothersill et al., 1998; Dowling et al., 2005). HaCaT cells have comparable survival to the control when they were directly exposed to low-dose radiation (0.1Gy γ-
rays or 0.1Bq/ml Ra-226) (Fernandez-Palomo et al., 2016; Shi et al., 2016). It is possible that progeny of these irradiation survivors retain the radioresistance or hormesis seen in their progenitors, and have increased clonogenic survival. This might also explain why HaCaT cells became adapted to radiation and had increased clonogenic survival if they were cultured in medium containing Ra-226 for more than 54 days (Shi et al., 2016). The hormetic cellular-level effects were also reported in fish cells exposed to concentrations of tritium corresponding to international drinking water standards, such as the increase in cell viability and the induced resistance to subsequent stress, but DNA damage occurred to the irradiated fish cells if they were tested with COMET assay (Stuart et al., 2016). In current study, there seems to be no evidence of detrimental effects in the progeny of Ra-226 irradiated HaCaT cells. But the increased survival in the descendants of irradiated survivors may still suggest an increased likelihood for carcinogenesis, and there might be some adverse effects at the molecular level, but these experiments did not test this.

Mitochondrial membrane depolarization may be involved in the induction of lethal mutation

Delayed apoptosis was demonstrated to be a significant cause of lethal mutation (delayed cell death), and apoptosis could occur in the distant progeny of irradiated cell (Lyng et al., 1996). Mitochondria have been reported to play a key role in the apoptotic process and their dysfunction has been shown to lead to the induction of apoptosis because of the leakage of inter-membrane apoptotic proteins and generation of reactive oxygen species through the depolarized mitochondrial membrane (Green and Reed, 1998; Ly et al., 2003). Normal mitochondrial membrane potentials are critical for mitochondria to regulate biochemical processes associated with cell survival and death. In the current work, mitochondrial membrane potential of the descendants of irradiated HaCaT and CHSE/F survivors was measured to see if there was involvement of potential apoptosis in the induction of lethal mutation. The result indicates that 9 days of low-dose α-radiation from Ra-226 could induce the depolarization of mitochondrial membrane in HaCaT cells. This is consistent with the previous research done in our laboratory which demonstrated that chronic low-dose radiation from Ra-226 was able to induce mitochondrial membrane depolarization in HCT116++/+ and HaCaT cells (Vo. et al., 2017). More importantly
mitochondrial membrane potential of the descendants of irradiated survivors was changed when compared to the descendants of unirradiated cells. Mitochondrial membrane potential value of the progeny of irradiated HaCaT survivors was found to be lower than that of the progeny of unirradiated cells at about 47 cell doublings after 9-days α-irradiation, and the reduction seemed to be dose-dependent (Figure 4d). The decreased mitochondria membrane potential also happened in the progeny of γ-irradiated HaCaT survivors at about 56 cell doublings and later (Figure 5d, e). The occurrence of mitochondrial membrane depolarization at this time may suggest the existence of apoptosis process in the progeny of irradiated survivors up to 47 cell doublings after irradiation. This agrees with the previous research which indicated the morphological abnormality of cells in the progeny of irradiated survivors up to 45 cell doublings after exposure (Lyng et al., 1996). But it is difficult to understand the occurrence of higher mitochondrial membrane potential value in some progeny of irradiated cells, such as the P2 of α-irradiated HaCaT cells (Figure 4b), P2 of γ-irradiated HaCaT cells (Figure 5b), and progeny of α-irradiated CHSE/F cells (Figure 10). The reason for higher MMP in the early passages is not known. But the ability of cell populations to recover from mitochondrial deficiencies has been reported in γ-irradiated and chronic low-dose Ra-226 irradiated human cells (Vo. et al., 2017).

5 Conclusions

The current study demonstrated that delayed reproductive cells death could be induced by the chronic low-dose high-LET radiation from Ra-226 in CHSE/F cells up to 60 cell doublings after irradiation, but the effect in HaCaT cells is not very obvious. In addition, the chronic α-radiation is more effective than γ-radiation in inducing delayed reproduction death in the progeny of irradiated cell survivors. Greater cell death happened and lasted longer in the descendant of α-irradiated cells than in the progeny of γ-irradiated survivor. This study could provide data for the study of lethal mutation induction by chronic high-LET radiation, and could be meaningful for estimating the influence of environmentally relevant level of Ra-226 on the non-human species in the environment.

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References


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Figure 1 Experimental process of lethal mutation induced by low-dose alpha irradiation from Ra-226 experiment. A: Experiment with progenies of HaCaT cells grown in medium containing Ra-
226; B: Experiment with progenies of HaCaT cells irradiated with gamma-rays; C: Experiment with progenies of CHSE/F cells grown in medium containing Ra-226; D: Experiment with progenies of CHSE/F cells irradiated with gamma-rays.
Figure 2 The clonogenic survival of HaCaT cells, (a) which were cultured in medium containing Ra-226 for 9 days (P0); (b–f) which were not directly irradiated and were the progenies (P1–P5) of cells surviving the Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 3 The clonogenic survival of HaCaT cells, (a) which were initially irradiated to 0-5 Gy γ-rays with Cs-137 (P0); (b-f) which were not directly irradiated and were the progenies (P1-P5) of cells surviving the γ-ray radiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 4 Relative mitochondrial membrane potential (MMP) in HaCaT cells, (a-e) at P1-P5 progenies of cells surviving Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 5 Relative mitochondrial membrane potential (MMP) in HaCaT cells, (a-e) at P1-P5 progenies of cells surviving γ-rays radiation with Cs-137. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 6 Relative number of HaCaT cells from one cell, (a) which were cultured in medium containing Ra-226 for 9 days and continued to be cultured in normal medium for 5 days (at P1); (b-e) number of cells counted at P2-P5 progenies of cells surviving the Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 7 Relative number of HaCaT cells from one cell, (a) which were initially irradiated to 0-5 Gy γ-rays with Cs-137 and continued to be cultured for 14 days (at P1); (b-e) number of cells counted at P2-P5 progenies of cells surviving the γ-ray radiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 8 The clonogenic survival of CHSE/F cells, (a) which were cultured in medium containing Ra-226 for 28 days (P0); (b-f) which were not directly irradiated and were the progenies (P1-P5) of cells surviving the Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 9 The clonogenic survival of CHSE/F cells, (a) which were initially irradiated to 0-5 Gy γ-rays with Cs-137 (P0); (b-c) which were not directly irradiated and were the progenies (P1-P2) of cells surviving the γ-ray radiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 10 Relative mitochondrial membrane potential (MMP) in CHSE/F cells, (a-e) at P1-P5 progenies of cells surviving Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 11 Relative mitochondrial membrane potential (MMP) in CHSE/F cells, (a-c) at P1-P3 progenies of cells surviving γ-rays radiation with Cs-137. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 12 Relative number of CHSE/F cells from one cell, (a) which were cultured in medium containing Ra-226 for 28 days and continued to be cultured in normal medium for 14 days (at P1); (b-e) number of cells counted at P2-P5 progenies of cells surviving the Ra-226 irradiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Figure 13 Relative number of CHSE/F cells from one cell, (a) which were initially irradiated to 0-5Gy γ-rays with Cs-137 and continued to be cultured for 42 days (at P1); (b-c) number of cells counted at P2-P3 progenies of cells surviving the γ-ray radiation. Error bars represent range, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher’s least significant difference (LSD) test. A significant change when compared to the respective sample in Control group is represented by * (p<0.05).
Highlights

- Chronic low-dose high-LET α-radiation from Ra-226 is effective in inducing lethal mutations.
- Decreased clonogenic survival did not occur in descendants of irradiated HaCaT survivors, and hormesis may be induced.
- Mitochondrial membrane depolarization may be involved in the induction of lethal mutation.