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The effects of chronic, low doses of Ra-226 on cultured fish and human cells

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ABSTRACT

Purpose: To determine the chronic low-dose radiation effects caused by α-particle radiation from ^{226}Ra over multiple cell generations in CHSE/F fish cells and HaCaT human cells.
Methods: CHSE/F cells and HaCaT cells were cultured in medium containing ^{226}Ra to deliver the chronic low-dose α-particle radiation. Clonogenic assay was used to test the clonogenic survival fractions of cells with or without being exposed to radiation from ^{226}Ra.
Results: The chronic low-dose radiation from ^{226}Ra does have effects on the clonogenic survival of CHSE/F cells and HaCaT cells. When CHSE/F cells were cultured in ^{226}Ra-medium over 9 passages for about 134 days, the clonogenic surviving fractions for cells irradiated at dose rates ranging from 0.00066 to 0.66 mGy/d were significantly lower than that of cells sham irradiated. For HaCaT cells grown in medium containing the same range of ^{226}Ra activity, the clonogenic surviving fraction decreased at first and reached the lowest value at about 42 days (8 passages). After that, the clonogenic survival began to increase, and was significantly higher than that of control cells by the end of the experimental period.
Conclusion: The chronic, low-dose high LET radiation from ^{226}Ra can influence the clonogenic survival of irradiated cells. CHSE/F cells were sensitized by the radiation, and HaCaT cells were initially sensitized but later appeared to be adapted. The results could have implications for determining risk from chronic versus acute exposures to radium.

1. Introduction

Radium-226 is a naturally occurring radionuclide in the decay chain of Uranium-238. It can cause severe damage to organisms if it is absorbed into the body because α-particles, which have a high linear energy transfer (LET), are mainly emitted in the decay chain. α-radiation is more effective than low LET radiation at causing biological damage and the relative biological effect (RBE) of it ranges from 1.6 to 21 depending on the endpoint used (Thomas et al., 2007; Howell et al., 1994; Franken et al., 2011). The high RBE is thought to be due to the fact that α-particle radiation can cause more clustered DNA double-strand breaks than sparely ionizing radiation and these breaks are more difficult to repair correctly (Blocher, 1988). In addition, because the chemical and physical properties of radium are similar to those of calcium, radium will compete with calcium after being absorbed and deposit in specific organs to form hot spots of high LET radioactive material (Priest et al., 1983).

Levels of ^{226}Ra in some areas are higher than the natural level because of human activities, such as uranium mining and milling, exploration and production of oil and gas and the radium production industry. Radium levels in the plants or animals in these areas are higher than normal (Giri et al. 2010; Pyle and Clulow, 1998; Brenner et al., 2007), and some adverse effects can be observed. For *Vicia cracca* populations growing in sites which were contaminated by uranium mill tailings and radium production wastes (^{226}Ra constituted 84.3–99% of the total internal irradiation), even when the absorbed dose was 0.2–0.3 Gy over about 120 days, the embryonic lethal mutation frequency was significantly higher and the germination capacity of seeds was significantly lower than those of control (Evseeva et al., 2009), so as to pine trees in the same sites (Geras'kin et al., 2011, 2013). The biomass of earthworms living in soil contaminated with α-emitting radionuclides (Radium-226 concentrations: 1506 Bq/kg dry weight) was significantly decreased, and their reproduction was inhibited (Lourenco et al., 2012). Studies in our group showed that Fathead minnow fed with the environmental level of ^{226}Ra exhibited transient growth perturbations, including the reductions in mean body mass and the disproportionate increase in body mass relative to fork length (Mothersill et al., 2013). The mechanisms underlying biological effects of low level ^{226}Ra radiation are complicated and unclear. A significant increase of DNA damage in the exposed earthworms (Lourenco et al., 2012) and changed ratios of DNA:

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RNA, DNA: protein and RNA: protein were found in fish (Mothersill et al., 2013) suggesting growth perturbations. Transcription of some genes was significantly up-regulated in Atlantic cod embryo cells exposed to environmentally relevant levels of ^{226}Ra and the induced oxidative stress and apoptosis may be trigger factors (Olsvik et al., 2012).

The enhanced level of ^{226}Ra in organisms in the environment leads to increased exposure to chronic, low-dose rate irradiation, which is important in Environmental Radiation Protection. At present because of the phenomena of bystander effects, adaptive responses and low-dose hypersensitivity, evaluating the risks of chronic low-dose radiation should not be done by extrapolating from data concerning acute often high dose radiation exposure (Seymour and Mothersill, 2000; Joiner et al., 1996; Mothersill and Seymour, 2004; Little, 2006; Morgan and Sowa, 2007; Frankenberg et al., 2006; Miura et al., 2002; Matsumoto et al., 2004; Varès et al., 2011). But studies about the effects induced by low-dose radiation are still rare, not to mention studies about the chronic, low-dose high LET radiation. The current study used fish cells (CHSE/F cell line) and human cells (HaCaT cell line) exposed to low-dose α-particle radiation from ^{226}Ra in medium for multiple cell-generations could provide a view to understanding the effect of this kind of radiation in a simple system where other confounding stressors were not present.

2. Materials and methods

2.1. Cell lines

CHSE/F fish cell line and HaCaT human cell line were used in the current study. CHSE/F (formerly known as CHSE-214) is a cell line derived from embryos of Common bluegill. This cell line has a typical epithelial-like morphology. During these experiments, they had an average plating efficiency of 41.4 ± 2.2% and a doubling time of about 127 hours. This cell line was obtained as a gift from Dr Niels Bols (University of Waterloo, Canada), and was frozen by previous students and stored in liquid nitrogen. The HaCaT cell line is an immortalized, nontransformed human keratinocyte cell line and was originally derived and characterized by Boukamp et al. from human skin keratinocytes (Boukamp et al., 1988). The cell line in our lab was obtained as a gift from Dr. Orla Howe (Dublin, Ireland), and was stored in liquid nitrogen. The average plating efficiency of the cells during these experiments was 51.4 ± 0.8% and the doubling time was about 23.6 hours. Cell cultures of these two cell lines were tested (Plasmo Test rep-pt1, InvivoGen, San Diego, CA) and confirmed to be mycoplasma free prior to use.

2.2. Cell culture

CHSE/F cells were cultured in Leibovitz's L-15 medium, supplemented with 12% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5 ml of 10,000 units of penicillin and 10,000 μg/ml of streptomycin (Gibco, Burlington, ON), 5 ml of 200 mM L-Glutamine (Gibco, Burlington, ON) and 25 mM Hepes buffer (Gibco, Burlington, ON). Cells stocks were maintained in T75 flasks with 30 ml of medium and subculture was routinely performed when cells were 80-100% confluent using a 1:1 solution of 0.125% trypsin and 1 mM EDTA at 19 °C for 8 mins. Cells were grown at 19 °C in an incubator without CO_2.

For HaCaT cells, RPM1640 medium, supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada), 5 ml of 10,000 units of penicillin and 10,000 μg/ml of streptomycin (Gibco, Burlington, ON), 5 ml of 200 mM L-Glutamine (Gibco, Burlington, ON), 0.5 g/ml hydrocortisone (Sigma-Aldrich, Oakville, ON), and 25 mM Hepes buffer (Gibco, Burlington, ON) was used for all experiments. Cells stocks were maintained within T75 flasks with 30 ml of medium. The subculture of HaCaT cells was routinely performed with a 1:1 solution of 0.25% trypsin and 1 mM EDTA at 37 °C for 8 mins. These cells were cultured in an incubator with 5% CO_2 at 37 °C. All experiments were performed in biosafety level 2 laminar flow cabinets.

2.3. ^{226}Ra medium

The radioisotope used in this study is Radium-226, supplied as neutralized radium nitrate by Eckert and Ziegler (Valencia, CA, USA). The carrier for the ^{226}Ra was 10 μg/ml Ba (as Ba nitrate). To prepare stock solutions, 100 ml L-15 or RPMI medium was mixed with 1000 Bq of ^{226}Ra solution, so the concentration of ^{226}Ra in this medium was 10,000 mBq/ml. The stock ^{226}Ra-medium was filtered into storage tubes. Then serial 1/10 dilutions were made to give final concentrations of medium with 10, 100, 1000 and 10,000 mBq/ml ^{226}Ra. The amount of ^{226}Ra in the medium is not sufficient to affect the role of other ingredients (including Calcium) in the medium. Respectively, the estimated concentration of Ba in the medium was about 0.00071 μg/l, 0.0071 μg/l, 0.071 μg/l and 0.71 μg/l. According to WHO guidelines for drinking water quality (2011), the guideline value of Barium concentration is 700 μg/l and the common concentrations in drinking-water are generally below 100 μg/l (WHO guidelines for drinking water quality, 4th edition). Therefore, the barium in the medium can be neglected.

2.4. Irradiation

For each cell line, there were five groups of cells: four groups of cells were cultured continuously in medium containing the different radium concentrations and one group of cells was cultured in control medium without radium. All groups of cells were cultured in T25flasks containing 5 ml medium in which the concentration of ^{226}Ra was 0, 10, 100, 1000 or 10,000 mBq/ml. The amount of radiation energy deposited per unit mass per unit time is known as dose rate. To get the dose rate of radiation induced by α-particles emitted from ^{226}Ra in the 5 ml medium, the radionuclide is assumed to be evenly distributed in the mixture of the medium and the cells, and the density of this mixture is assumed to be equal to water. The dose rate can then be calculated according to the following equation:

$$D_\alpha = C_{226Ra} \times V \times E_\alpha / m(mBq \cdot MeV/g)$$
$$= 1.38 \times 10^{-5} \times C_{226Ra} \times E_\alpha (mGy/d)$$

where D_α is the dose rate of radiation from ^{226}Ra in medium to the whole mixture. E_α is the average energy of α particles emitting from ^{226}Ra, 4.78 MeV. C_{226Ra} is the concentration of ^{226}Ra in the mixture (mBq/ml) and 1.38×10^{-5} is a conversion factor.

After calculation, the dose rate for each group was 0 mGy/d, 0.00066 mGy/d, 0.0066 mGy/d, 0.066 mGy/d or 0.66 mGy/d respectively. All five groups of cells were treated similarly. At the time of each subculture, a clonogenic assay was done to test the clonogenic survival of cells in each group, and the total number of cells in each flasks were determined to calculate the doubling time of cells during this period.

2.5. Clonogenic assay technique

Clonogenic assay technique described by Puck and Marcus (Puck and Marcus, 1956) was used for clonogenic survival analysis. Briefly, cells were detached from the flasks and were resuspended in medium. Then an aliquot of the cell suspension was counted using a Z2 Coulter particle count and size analyzer (Bechman Coulter Electronics, Mississauga Ontario, Canada) to determine the

number of viable cells. After that, appropriate number of cells were plated into each flask, and cell cultures were incubated for 4 weeks at 19 °C for CHSE/F cell line or 9 days at 37 °C for HaCaT cell line. Flasks were checked periodically for growth. When colonies in the control flasks were visible to the naked eye, the cells were stained with 20% carbol fuchsin in water (VWR, Bridgeport, NJ, USA) and colonies with 50 cells or more were counted.

2.6. Cell population doubling time

The approximate time it takes for CHSE/F cells and HaCaT cells to double in number during every passage was determined through counting the number of cells at the beginning and at the end of each passage. Any changes in the doubling time may indicate possible alternations in the viability of cells. At the time of each subculture, T25 flasks with 5 ml of medium were set up for cells in each group, and appropriate number of cells (N_0) was plated into each flask. After a period of time (T hours), when cells were 80-100% confluent, cells were detached from the flasks and were resuspended in medium. Then an aliquot of the cell suspension was counted using a Z2 Coulter particle count and size analyzer (Bechman Coulter Electronics, Mississauga Ontario, Canada) to determine the number of viable cells. Because the volume of the suspension was known, the total number of cells in the flask was determined (N). The doubling time of cells in this passage can be calculated according to the following equation:

$$\text{doubling time} = T \times [\ln(2)/\ln(N/N_0)].$$

2.7. Statistical analysis

All data are expressed as means ± SEM (the standard error of the mean).The effect of chronic, low-dose radiation from ^{226}Ra on the clonogenic survival of CHSE/F cells or HaCaT cells was 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. Radium effects on CHSE/F cell line

3.1.1. Radium effects on clonogenic survival of CHSE/F cells

The overall plating efficiency of the CHSE/F cells used in this study was 41.4 ± 2.2%. The plating efficiency of CHSE/F cells in control groups was respectively 33.2 ± 1.8%, 36.9 ± 1.0%, 41.8 ± 3.3%, 44.6 ± 3.3%, 40.4 ± 1.0% and 56.2 ± 1.6% after being cultured in medium for 12, 36, 50, 64, 99 and 134 days. The total dose of radiation that CHSE/F cells received over multiple generations in the experiments was shown in Table 1. Clonogenic surviving fractions of cells with or without being irradiated by ^{226}Ra in medium are shown in Fig.1. And in Fig.2, the percentage of change in clonogenic survival between cells in each ^{226}Ra-medium group and control group is demonstrated. At early time points, ^{226}Ra didn't induce significant cell killing in CHSE/F cells in each ^{226}Ra-medium group compared with in the control, even though there was the trend towards lower clonogenic surviving fractions for cells in ^{226}Ra-medium group. By 50 days, CHSE/F cells cultured in 0.66 mGy/d ^{226}Ra-medium began to show significant cell killing, and the clonogenic surviving fraction was 81.63 ± 4.09%, p=0.003. But the sensitizing effect disappeared in the following passages. When cells were cultured in ^{226}Ra-medium for 134 days, significantly decreased clonogenic survival began to appear in most cells. At that time, the clonogenic survival fraction for cells exposed to α-particle radiation at the dose rate of 0.00066 mGy/d was about 77.15 ± 2.52%, $p < 0.001$, which was 22.85% lower than that of control cells. The clonogenic surviving fractions of cells exposed to 0.066 mGy/d and 0.66 mGy/d α-particle radiation were 79.43 ± 2.26%, p=0.001 and 84.56 ± 3.26%, p=0.012.

Table 1
The total dose (mGy) of radiation that CHSE/F cells received from ^{226}Ra over multiple generations.

Dose rate/Days	12 d	36 d	50 d	64 d	99 d	134 d
Control (0 mGy/d)	0	0	0	0	0	0
0.00066 mGy/d	0.00792	0.02376	0.033	0.04224	0.06534	0.08844
0.0066 mGy/d	0.0792	0.2376	0.33	0.4224	0.6534	0.8844
0.066 mGy/d	0.792	2.376	3.3	4.224	6.534	8.844
0.66 mGy/d	7.92	23.76	33	42.24	65.34	88.44

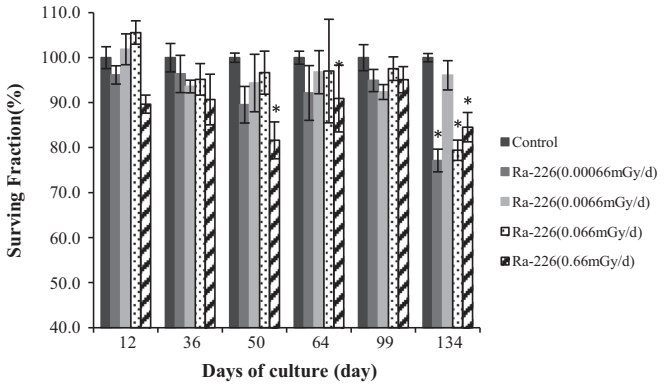

Fig. 1. The clonogenic surviving fraction of CHSE/F cells cultured in medium with or without ^{226}Ra. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0 mBq/ml) group is represented by *($p < 0.05$).

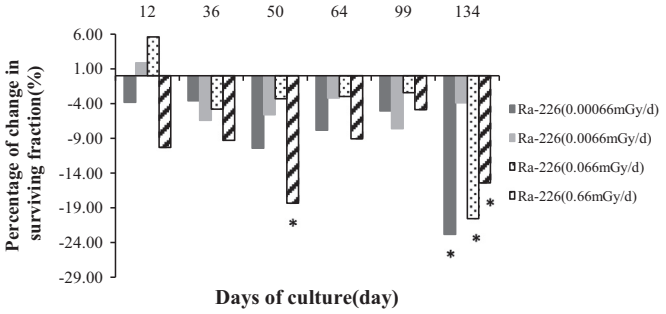

Fig. 2. Percentage of change in clonogenic surviving fractions of CHSE/F cells cultured in medium with or without ^{226}Ra, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0 mBq/ml) group is represented by * ($p < 0.05$).

3.1.2. Radium effects on CHSE/F cell population doubling time

The average doubling time of CHSE/F cells in control group during the whole experiment was about 127 hours. And for the six selected periods in the experiment, the tested doubling time was respectively 123 h, 140 h, 149 h, 135 h, 145 h and 110 h. The doubling time of CHSE/F cells during every passage was normalized to that of cells during respective passage in the control group to offset the experimental difference, and the result was

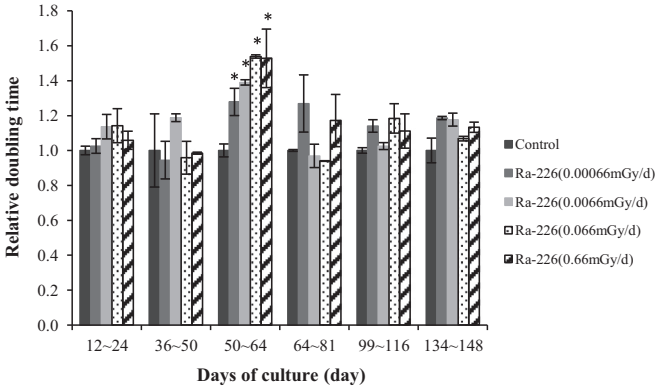

Fig. 3. The doubling time of CHSE/F cells cultured in medium with or without ^{226}Ra. Error bars represent SEM, n=6. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change compared to Control (0 mBq/ml) group is represented by *($p < 0.05$).

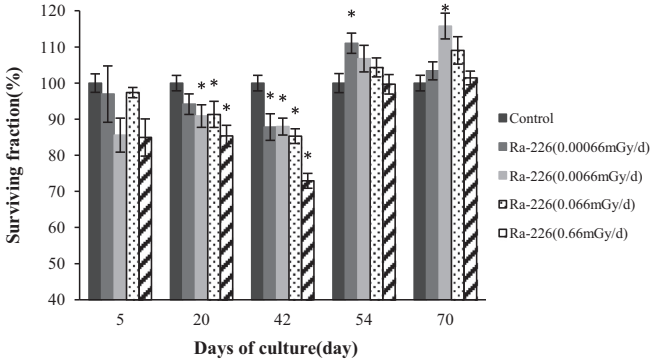

Fig. 4. The clonogenic survival fractions of HaCaT cells cultured in medium with or without ^{226}Ra. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0 mBq/ml) group is represented by * ($p < 0.05$).

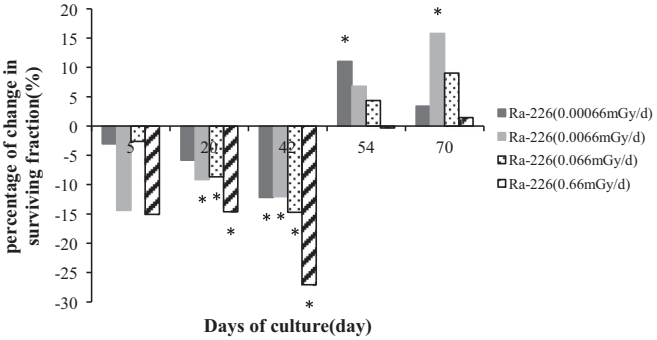

Fig. 5. The percentage of change in clonogenic surviving fractions of HaCaT cells cultured in medium with or without ^{226}Ra, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test. A significant change when compared to the respective sample in Control (0 mBq/ml) group is represented by *($p < 0.05$).

demonstrated in Fig. 3. The doubling time of CHSE/F cells grown in medium containing ^{226}Ra was almost the same as that of cells grown in normal medium except for the period of the 5th passage when cells were cultured in medium from 50 days to 64 days. During this period, the doubling time of cells in the four ^{226}Ra-treated groups was respectively 1.28 ($p=0.008$), 1.39 ($p=0.003$), 1.54 ($p < 0.001$) and 1.53 ($p < 0.001$) times as long as that of cells in control group. Cell proliferation took longer time for cells cultured in ^{226}Ra-medium during that cell passage.

3.2. Radium effects on HaCaT cell line

3.2.1. Radium effects on clonogenic survival of HaCaT cells

The overall plating efficiency of HaCaT cells used in this study was $51.4 \pm 0.8\%$, and at the five selected time points, the plating efficiency of cells in control group was $51.2 \pm 1.4\%$, $51.7 \pm 2.6\%$, $57.1 \pm 2.7\%$, $50.8 \pm 1.4\%$ and $48.7 \pm 1.4\%$, which was pretty stable during the experiment period. The total dose of radiation received by HaCaT cells over multiple generations in the experiment was shown in Table 2. Fig.4 shows the clonogenic surviving fraction of cells with or without exposure to ^{226}Ra in the experiment. The percentage change in clonogenic surviving fractions for cells in each ^{226}Ra-medium group and the control group is shown in Fig.5. The clonogenic survival of HaCaT cells cultured in ^{226}Ra-medium decreased at first and reached the lowest value after being cultured over 8 passages for 42 days. In the next passage the clonogenic survivals began to rise up and kept increasing to the end of the experiment. Decreased clonogenic survival began to happen when HaCaT were cultured in ^{226}Ra-medium for 20 days, except for those grew in medium with the lowest concentration of ^{226}Ra. After being cultured for 42 days, the clonogenic surviving fraction of all cells in ^{226}Ra-medium group reached the lowest value: $87.82 \pm 3.68\%$, $p=0.006$, $87.97 \pm 2.34\%$, $p=0.006$, $85.31 \pm 2.03\%$, $p=0.001$ and $72.95 \pm 2.06\%$, $p < 0.001$. Cells irradiated by 0.66 mGy/d radiation had the lowest clonogenic survival fraction, which was about 27.05% lower. After that, cells began to show the sign of getting adapted to the low-dose radiation, and their clonogenic survival began to increase. The value was $111.05 \pm 2.80\%$ ($p=0.012$) for cells exposed to 0.00066 mGy/d radiation for 54 days and $115.80 \pm 3.572\%$ ($p=0.001$) for cells irradiated by 0.0066 mGy/d radiation for 70 days. The increased clonogenic survival also happened to cells irradiated at other dose rates, but didn't reach significance.

Table 2
The total dose (mGy) of radiation that HaCaT cells received from ^{226}Ra over multiple generations.

Dose rate/Days	5 d	20 d	42 d	54 d	70 d
Control (0 mGy/d)	0	0	0	0	0
0.00066 mGy/d	0.0033	0.0132	0.02772	0.03564	0.0462
0.0066 mGy/d	0.033	0.132	0.2772	0.3564	0.462
0.066 mGy/d	0.33	1.32	2.772	3.564	4.62
0.66 mGy/d	3.3	13.2	27.2	35.64	46.2

3.2.2. Radium effects on HaCaT cell population doubling time

The average doubling time of HaCaT cells in control group over the whole experiment was about 23.6 h, and for the five selected time periods, the doubling time was 19.8 h, 21.1 h, 25.3 h, 26.1 h and 24.2 h. In order to offset the experimental difference between each cell passage, the doubling time of HaCaT cells during every passage was normalized to that of cells in the control group of that passage, and the result was demonstrated in Fig. 6. During each passage, the doubling time of HaCaT cells grown in ^{226}Ra-medium was not different from that of cells grown in normal medium. It seems that the chronic radiation from ^{226}Ra didn't affect the growth rate of HaCaT cells.

4. Discussion

Generally speaking, the chronic, low-dose radiation from

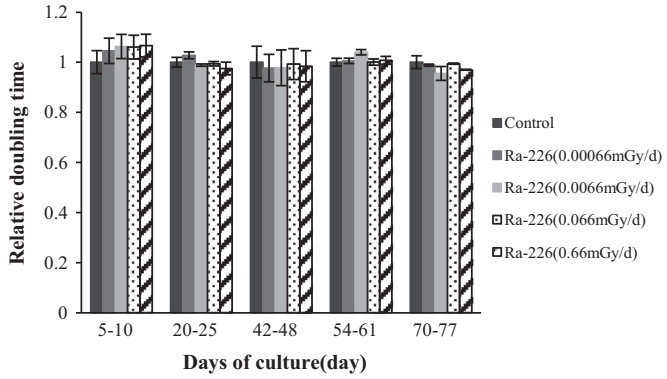

Fig. 6. The doubling time of HaCaT cells cultured in medium with or without ^{226}Ra. Error bars represent SEM, n=9. Analysis was performed using two-way Analysis of Variance (ANOVA) method, and Post-hoc testing was performed with Fisher's least significant difference (LSD) test.

environmental level of ^{226}Ra may not have obvious influence on the organisms in the natural environment. In our experiment, after being cultured in ^{226}Ra-medium over multiple generations for a long time period, the clonogenic survivals of both CHSE/F cells and HaCaT cells were affected at some time points, but the doubling time of both cell lines was not influenced. So it can be assumed that even though the chronic, low-dose radiation can affect the colonies-forming ability of a single cell, but the impact can be reduced when a large number of cells were present.

For CHSE/F cells, decreased clonogenic survival happened earliest when cells were cultured in 0.66 mGy/d ^{226}Ra-medium for 50 days (accumulated dose was 33 mGy). When they were cultured in ^{226}Ra-medium for about 134 days, decreased clonogenic surviving fraction happened to most cells (Fig.2). Among the sensitized cells, the lowest dose of radiation they received was 0.0884 mGy (concentration of ^{226}Ra in medium was 10 mBq/ml), and the clonogenic survival was 22.85% lower than that of the control. Because the plating efficiency in this time point was higher than other time points, the decreased clonogenic survival of CHSE/F cells in ^{226}Ra-medium group might be partly because of the increased plating efficiency. Compared to CHSE/F cells, the sensitizing reaction of HaCaT cells to the radiation from ^{226}Ra happened earlier. Cells cultured in ^{226}Ra-medium for about 20 days (4 passages) began to show significantly decreased clonogenic survival except for cells in the lowest ^{226}Ra concentration group. After being cultured in ^{226}Ra-medium for 42 days, all cells had significantly decreased clonogenic survivals than control cells, and the accumulated dose of radiation received by them was respectively 0.028 mGy, 0.28 mGy, 2.8 mGy and 28 mGy. In addition, the sensitizing response of HaCaT cells seemed to be positively relevant to the accumulated dose of radiation in each group. In another word, cells grown in ^{226}Ra-medium for 42 days had lower clonogenic survival than cells cultured in medium containing the same concentration of ^{226}Ra for 20 days (in Fig.5).

The result that low-dose α-radiation have influence on cells' survival is consistent with the gene transcription experiments for Atlantic cod embryo cells, which found that some gene transcriptional levels were changed even when the ^{226}Ra concentration was as low as 2.11 mBq/ml (Olsvik et al., 2012). Chromosome aberration were also reported to be increased in human blood lymphocytes exposed to 0.01, 0.02 and 0.03 mGy α-radiation from Radon (Hamza and Mohankumar, 2009), as well as the frequency of sister chromatid exchanges in Chinese hamster ovary (CHO) cells exposed to 0.31 mGy α-radiation from ^{238}Pu (Nagasawa and Little, 1992) and in human diploid lung fibroblasts (HFL1) cells exposed to 4–129 mGy α-radiation (Deshpande et al., 1996). HPRT point mutations could also be increased by 5 mGy α-radiation (Huo et al., 2001). It was reported that the transmissible genetic instability induced by α-radiation would lead to the occurrence of chromosomal aberrations among progeny cells after many generations of replication (Kadhim et al., 1992). Because the cells we used underwent multiple cell passages in the radioactive ^{226}Ra-medium, we speculate that the low-dose α-radiation induced the genetic instability and aberrations in cells at first, and as time went on, these changes may be developed and expressed over time as cell killing.

But our result that decreased clonogenic survival could be induced by such low doses of α-radiation in cells is different from another study, which found that no evidence of cell killing was observed in CHO cells exposed to α-radiation at doses up to 4.9 mGy (Nagasawa and Little, 1992). The possible reason might be the more severe bystander effect induced in our cells. In that study, they irradiated CHO cells from below through the Mylar base dishes by 3.7 MeV α-particles from ^{238}Pu for only 0.125–2 seconds, and they found that if CHO cells were irradiated for 0.125 second for 0.31 mGy, 30% of the cells showed an increased frequency of SCE, even though less than 1% of cell nuclei were actually traversed by an α-particle. This means that communication among cells occurred and DNA damage was not the direct inducement (Nagasawa and Little, 1992). In our work, the cells were directly grown in the radioactive medium and were continuously exposed to α-radiation for multiple passages over dozens of days. We speculate that the bystander effect in our cells might be accumulated over the long time period, and signals from damaged cells and previously-influenced cells can affect more cells as a chain reaction (Mothersill and Seymour, 1998; Seymour and Mothersill, 2000; Lyng et al., 2000).

The bystander effect induced by α-radiation may also be the reason why decreased clonogenic survival happened earlier to HaCaT cells than to CHSE/F cells. HaCaT cells are sensitive to bystander signals, and signals from damaged cells can reduce clonogenic survival in unirradiated cells (Ryan et al., 2009). The adverse influence of α-radiation on cells' clonogenic survival and the cell killing induced by bystander signal might be additive, so extreme low-dose radiation could induce obvious cell killing in HaCaT cells in current work. On the contrary, CHSE/F cells showed increased cloning efficiency in unirradiated cells treated by irradiated cell conditioned medium (O'Neill-Mehlenbacher et al., 2007). The cell killing caused by α-particle radiation and the increased clonogenic survival induced by bystander effect might offset each other. So for CHSE/F cells, higher doses of radiation were needed to induce significantly decreased clonogenic survival.

The sensitizing response of CHSE/F cells and HaCaT cells to ^{226}Ra might be because of the apoptosis induced by the low-dose α-radiation. Some apoptotic cells were found in stained CHSE/F cells and HaCaT cells cultured in ^{226}Ra-medium. It was reported that 0.29 mGy α-radiation was sufficient to produce an observable increase of apoptosis in cocultured nonirradiated transformed cells (65 hours of coculture) through intercellular induction of apoptosis involving ROS/NOS and TGF-β, and the apoptosis saturated at very low-dose radiation (25 mGy for α-particles). In addition, the level of apoptosis continued to increase as cells remained in coculture, and eventually the vast majority of transformed cells were removed by the induced intercellular apoptosis (Portess et al., 2007). Gene expression related to apoptosis, such as bax, bcl-2 and bcl-xl, was also reported to be changed by low-dose α-radiation from ^{222}Rn 0.6–8.3 mGy) in human breast cancer cells (MCF-7) (Soto et al., 2006). In addition, genes related to cell cycle arrest, DNA replication and repair were differentially expressed in human lung epithelial cells exposed to 0.3 and 0.9 Gy of α-particle radiation (Chauhan et al., 2012). So the decreased clonogenic survival in cells in current work could be explained by the induction of apoptosis. The low-dose radiation received by CHSE/F cells and

HaCaT cells from ^{226}Ra in medium might change the micro-environment of the cells and induce higher oxidative stress. Then cell apoptosis was induced through intercellular signal pathways. The cellular defense through killing cells with damaged unrepaired DNA and cells with tumorigenesis possibility could prevent passing mutations onto offspring and decrease the risk of tumorigenesis (Real et al., 2004; Mothersill and Seymour, 2004; Portess et al., 2007). From this point, the sensitizing effect of ^{226}Ra on CHSE/F cells and HaCaT cells might be a protective mechanism. In the following work, the apoptosis of cells and genes related with apoptosis and DNA repair will be tested to see whether or not apoptosis is the main reason for the sensitizing effect.

An unexpected result was that after being cultured in ^{226}Ra-medium for 42 days, HaCaT cells showed the sign of getting adapted to the low-dose radiation. The clonogenic surviving fractions of cells in all ^{226}Ra-medium groups began to increase, among which, cells irradiated with 0.00066 mGy/d α-radiation for 54 days and cells irradiated with 0.0066 mGy/d α-radiation for 70 days had significantly increased clonogenic survival (11.05% higher and 15.80% higher), (shown in Fig.5). We suggest that this is a kind of protective adaptive effect, the hormetic response, in which, low-dose radiation can lead to increasing clonogenic survival and cloning efficiency (Wang and Cai, 2000; Redpath et al., 2001; Scott, 2004). There appears to be two types of adaptive protection. One is to remove damaged cells by inducing apoptosis mentioned above (Portess et al., 2007) and terminate differentiation to reduce genomic instability and tumorigenesis. The other one is to prevent and repair DNA damage to keep cells alive and functioning properly (Feinendegen, 2005). The induction of apoptosis usually happens hours to days after acute low-dose low LET radiation, and DNA repair and cell proliferation usually happens days to weeks after the radiation (Feinendegen et al., 2002). The duration of the effectiveness of each mechanism and the time interval between energy deposition events of radiation in the target determine which mechanism of adaptive protection prevails and to what degree damage or protection reach (Feinendegen, 2005). So we speculate that in our experiment, the induction of apoptosis prevailed at first, so the damaged cells and the signal-influenced cells were eliminated, which led to the decreased cell clonogenic survival. As time went on, DNA repair and cell proliferation prevailed, and cell clonogenic survival showed the trend of increase.

In current work, the doubling times of CHSE/F cells and HaCaT cells were not obviously influenced by the low-dose α-radiation from ^{226}Ra. Only in one time period, when CHSE/F cells were cultured in ^{226}Ra-medium from 50 days to 64 days, their doubling time was significantly longer than that of control cells. Because CHSE/F cells cultured in 0.66 mGy/d 226Ra-medium for 50 days had decreased clonogenic survival, this may indicate that the decreased cell clonogenic survival affected the repopulation of cells. No influence of ^{226}Ra on the doubling time of cells means that the chronic, low-dose radiation could not influence the cell proliferation. Cells may get adapted to ^{226}Ra in the medium, and could keep the normal proliferation. But the colony-forming ability of cells was affected. This might be because of the different number of ^{226}Ra atoms surrounding each cell. In clonogenic assay, cells were sparsely planted in each flask, and in cell culture for doubling time, much more cells were seeded in each flask. Even though cells in the whole flask received the same dose of radiation, in cell culture for doubling time, the surrounding for every cell were much less. Because of the similar chemical and physical characteristics of ^{226}Ra and calcium, the ^{226}Ra and calcium in the medium would compete to enter into cells. So more ^{226}Ra atoms would enter into each cell to form internal radiation when cells were sparsely planted. In addition, a cell is more possible to be hit by α-particles because no other cells available to block the hit. So ^{226}Ra in medium could have more obvious effect on cells in clonogenic survival analysis than in doubling time analysis. Of course, it is also possible that maybe a separate set of factors determines the population doubling rather than the ability of forming colonies. So further experiments such as analyzing the size of the colonies, measuring the apoptosis and testing the change of calcium flux in the cells are needed to find out the reason.

Overall, the results provide some data about the effects caused by chronic low dose of high LET radiation in the environmentally relevant range. This may be important for the protection of human and non-human biota in the environment. CHSE/F cell line is a fish cell line, which is radiation resistant compared with some human cell lines, so their reaction to the low dose radiation from ^{226}Ra may not be very great. The clonogenic survival fraction of HaCaT cells decreased at first and then increased as time of exposure to ^{226}Ra-medium increased suggesting that an adaptive mechanism was induced.

5. Conclusions

In this study, the chronic low-dose radiation from ^{226}Ra does have effects on the clonogenic survival of CHSE/F cells and HaCaT cells, but the influence on the doubling time was not so obvious. When CHSE/F cells were cultured in ^{226}Ra-medium for about 134 days, their clonogenic surviving fractions were significantly lower than that of control cells. CHSE/F cells treated with 0.66 mGy/d radiation from ^{226}Ra in medium for 50 days also had decreased clonogenic survival. The doubling time of CHSE/F cells treated with ^{226}Ra was almost the same as that of control cells except for the period when cells were cultured in medium containing ^{226}Ra from 50 days to 64 days. Clonogenic survival of HaCaT cells treated with ^{226}Ra in medium decreased at first and then increased as the time of culturing in ^{226}Ra-medium increasing. The doubling time of HaCaT cells was not influenced by ^{226}Ra in the medium. This study could be meaningful for radiation protection of human and non-human biota to the chronic, low-dose high LET radiation in the environment.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content of the paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.04.009.

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