The response of human mesenchymal stem cells to internal exposure to tritium β-rays

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ABSTRACT

There is no doubt that estimating the exposure risk of external and internal low-dose radiation is an imperative issue in radiobiological study. Human mesenchymal stem cells (hMSCs) are multipotent and self-renewing, supporting the regeneration of damaged tissue, including tissue damaged by radiation. However, the responses of hMSCs to internal exposure to radionuclides are still insufficiently understood. In order to evaluate the adverse effects produced by internal exposure to tritiated water (HTO) at a low dose, hMSCs were exposed to \(2 \times 10^7\) Bq/ml HTO, and the biological effects after the exposure were examined. Apoptosis and DNA double-strand breaks (DSBs) were assayed to analyze the cellular response to the damage induced by HTO. Slight enhancement of apoptosis was found after treatment, except at the dose of 9 mGy. The number of DSBs at 24 h post-irradiation showed that the DNA damage was able to be efficiently repaired by the hMSCs. Moreover, the increasing proportion of the cell population in S phase proved that the persistence of residual γH2AX foci at lower concentrations of HTO was attributable to the secondary production of DSBs in DNA replication. Our work adds to the available data, helping us understand the risk of stem cell transformation due to internal exposure and its correlation with low-dose radiation-induced carcinogenesis.

Keywords: stem cell; tritiated water; internal exposure; low-dose radiation

INTRODUCTION

Through extensive study of carcinogenesis, increased evidence has supported the hypothesis that cancers contain a stem cell–like population of cells [cancer stem cells (CSCs)] that can promote the process of oncogenesis [1–3]. CSCs share many characteristics with normal stem cells, including indefinite potential for self-renewal and the capacity for sustaining cell growth [4, 5]. In contrast with differentiated cells losing their pluripotent potential, the molecular response of stem cells to genotoxic damage is highly associated with tissue homeostasis and cellular carcinogenesis [6]. In addition, the long lifespan of stem cells allows accumulation of additional mutations in specific genes, which has led to the proposition that stem cells are related to the cells-of-origin of some human cancer occurrences [7, 8]. Therefore, understanding stem cell behavior after ionizing irradiation can help us to figure out the relationship between stem cells responding to radiation damage and cancer induction. An abundance of studies on exposure to external radiation have been carried out to investigate the damaging effects and mechanisms of the response of stem cells to low-dose ionizing radiation [9].

Based on the findings, the relationship between the mutation of stem cells and cancer induction as a result of low-dose radiation has the potential to be clarified. However, in contrast to external radiation, there are more uncertainties associated with cancer risk estimation in relation to internal exposure to radionuclides, because the dose measurement and the reconstruction of exposure dose to internal emitters is more difficult [10]. Moreover, the damaging effects induced by internal exposure depend not only on the rate of radioactive decay, but also on its chemical characteristics. The complex interaction and distribution of radionuclides in organs and tissues make it difficult to understand the injury occurrence process when human beings are exposed to internal radiation. Further study is needed on the risks to health from internally deposited radionuclides.

Tritium is a natural radioactive isotope of hydrogen, and the primary way in which it can be a biological hazard is through internal exposure to it in the form of tritiated water (HTO) [11]. HTO can quickly become homogeneously distributed throughout the whole body and has a mean biological half-time of 9.5 days [12].
energy of beta particles emitted from tritium can be absorbed by adjacent biomolecules and result in their ionization or activation, which subsequently triggers a functional change [13]. Long-term retention of organically bound tritium (OBT) has the potential to increase the occurrence of stochastic effects [14]. Hagger et al. found concentration-dependent effects for the induction of cytogenetic damage, developmental abnormalities and mortality, when 1-h-old embryo-larvae of marine mollusc Mytilus edulis were exposed to a range of concentrations (0.37–370 kBq/ml) of HTO with a delivered dose of between 0.02 and 21.41 mGy [15]. Limited epidemiological studies indicate that after long-term chronic exposure to tritium, there is a higher biological effectiveness of aberration frequency in the peripheral blood lymphocytes, compared with that following exposure to gamma irradiation [16]. Few studies have investigated the detrimental effects on stem cells of exposure to low-dose radiation, especially internal exposure to low-dose radiation. Therefore, studying the response of cells to HTO of different radioactivity levels, and illuminating the mechanisms involved in this cell behavior, will play an important role in determining whether or not low-radioactivity HTO increases the risk of cancer occurrence. Because tritium is homogeneously distributed throughout the whole body, and because bone marrow is one of the most radiosensitive tissues [17, 18], bone marrow-derived mesenchymal stem cells (MSCs) were studied in the present work. Dose–response experiments involving low (9, 53 and 88 mGy) and intermediate (530 and 880 mGy) doses were analyzed. DNA damage, represented by double-strand breaks (DSBs), was quantified using immunofluorescence.

MATERIALS AND METHODS

Cell culture
All experiments were performed with hMSCs, and cells were cultured in basal medium supplied with 7% fetal bovine serum, 15 ng/ml rhIGF-1, 125 pg/ml RhFGF-b, 2.4 mM L-Alanyl-L-Glutamine (ATCC PCS-500-041).

Radiation protocol
HTO with a radioactivity of 2 × 10^8 Bq/ml HTO (provided by the Research Institute of Nuclear Physics and Chemistry, China Academy of Engineering Physics, China) was diluted to various concentrations. Cells were incubated in medium supplied with the same concentration of HTO (2 × 10^7 Bq/ml HTO) for various times (approximately 10 min, 60 min, 100 min, 600 min or 1000 min) to obtain different doses (corresponding to 9 mGy, 53 mGy, 88 mGy, 530 mGy and 880 mGy, respectively), or exposed to different concentrations of HTO with the same culture time (exposed to 2×10^7 Bq/ml, 2×10^8 Bq/ml or 2×10^9 Bq/ml HTO, for 1000 min). After incubation, cells were rinsed with medium twice to remove residual HTO from the petri dish. The removal of HTO from the cell cultures was monitored by measuring the radioactivity of the rinse medium with a liquid scintillation analyzer (PerkinElmer, Tri-Carb 3100TR).

The average absorbed dose was obtained using the following formula (Equation 1) [19]:

$$D_\beta = 3.29 \times 10^{-3} \cdot C \cdot W (\text{Gy/h})$$  

(1)

In this formula, $D_\beta$ represents the dose rate of beta particle radiation, C is the specific radioactivity of HTO (MBq/ml), and $3.29 \times 10^{-3}$ is the conversion factor ($1 \times 10^6 \text{decay} \cdot \text{s}^{-1} \cdot \text{MBq}^{-1} \times 3.6 \times 10^{13} \text{s}^{-1} \cdot 5.7 \times 10^7 \text{eV-decay}^{-1} \times 1.602 \times 10^{-9} \text{eV}^{-1} \times 1 \text{Gy} \cdot \text{j}^{-1} \cdot \text{kg}^{-1} \times 1 \times 10^3 \text{g} \cdot \text{kg}^{-1}$). W represents the water content of the cell, and the value of 0.80 has often been used for W in cultured mammalian cells.

Cell cycle analysis
Irradiated cells were harvested at 3 h and 24 h post HTO treatment separately, and fixed in dehydrated ethanol overnight at 4°C. Samples were stained with 10 µg propidium iodide (PI) solution, and >10,000 cells were detected via flow cytometry (Beckman CytoFLEX FCM). The cell-cycle distribution fractions were evaluated from the DNA content histograms using FlowJo software.

Apoptosis
Apoptotic cells were analyzed through flow cytometry via the staining of Annexin V and PI as previously described [20]. More than 10,000 cells were examined via flow cytometry and analyzed by FlowJo software.

Immunofluorescence
The gamma H2AX staining method was performed as previously described [21]. More than 100 cells were randomly counted in each sample by an investigator blind to the experimental conditions.

Statistic analysis
All data presented are representative of at least three independent experiments. Student’s t-test was used when mean differences between the groups of treatment and control were evaluated by SPASS software. For all comparisons, a value of $P < 0.05$ was defined as significant.

RESULTS

Apoptosis of hMSCs induced by acute exposure to 2 × 10^7 Bq/ml HTO
To explore the adverse consequences of short-term exposure to HTO, the level of apoptosis in hMSCs was measured at 3 h and 24 h post incubation with 2×10^7 Bq/ml HTO (Fig. 1). After acute exposure to HTO, apoptotic cells were measured via flow cytometry and evaluated as the population having a high expression of fluorescein isothiocyanate (FITC)-labelled Annexin V accompanied by the negative red fluorescence of PI. Compared with the sham-irradiated group, an increase of Annexin V and PI as previously described [22].

Quantification of γH2AX foci produced by different doses after acute exposure to 2 × 10^7 Bq/ml HTO
The average foci number per cell induced by different doses of irradiation was manually scored and is presented in Fig. 2. The analysis of the average number of foci showed that, even for the dose is as low as 9 mGy, the mean foci number per cell clearly increased in contrast to the control group. Interestingly, the foci number for
DNA damage response to short exposure to HTO with various levels of radioactivity

Further, to investigate the influence of various levels of radioactivity on DSB induction, cells were exposed to HTO of varying radioactivity but the same incubation time. The results showed that the DSB number increased with increasing radioactivity, and the number of foci produced by $2 \times 10^7$ Bq/ml HTO treatment was efficiently returned to the control level (Fig. 3). In addition, the disappearance rate of the remaining foci in $2 \times 10^5$ Bq/ml and $2 \times 10^6$ Bq/ml HTO was slower than that in $2 \times 10^7$ Bq/ml HTO. The assessment of cell cycle distribution indicated that, after exposure to $2 \times 10^6$ Bq/ml and $2 \times 10^5$ Bq/ml HTO, the proportion of cells in S phase substantially increased, and there was a simultaneous decrease in the number of cells in G1 and G2 phases (Fig. 4).

**DISCUSSION**

Stem cells play a key role in maintaining organism homeostasis due to their potential for self-renewal and multiple types of differentiation [32]. Although abundant studies have investigated the biological effects of stem cells exposed to low-dose radiation, little is known about internal exposure to radionuclides [9, 23]. HTO is one of the radioactive wastes discharged from nuclear power plants. Due to the low penetration depth of tritium β-rays, the hazardous effects of HTO are considered to only occur when it is absorbed into the body through the ingestion of drinking water or food containing HTO. Once taken in, the distribution of HTO between the organs and tissues is quite uniform, and all cells have the potential to be exposed to tritium β-rays. Bone marrow is one of the most radiosensitive tissues and under situations of internal exposure to HTO, how hMSCs respond to this damage needs to be understood. Hence, the detrimental effects of low-dose exposure to HTO on hMSCs were studied.

Our findings regarding the lethal effects of short-term exposure to $2 \times 10^7$ Bq/ml HTO indicated that there was no significant increase in apoptosis at the low dose of 9 mGy, but that there was a slight increase at or above the dose of 53 mGy (Fig. 1). Alessio et al. found that at a low dose of X-ray irradiation, the contribution to apoptosis was marginal and the main consequence was the triggering of senescence, which was progressively enhanced between 40 mGy and 2000 mGy [24]. Moreover, even for doses up to 20 GY, hMSCs prefer to enter senescence rather than apoptosis [25, 26]. This minimal cellular apoptosis is associated with high expression levels of anti-protein BCL-XL and BCL-2 and low levels of pro-apoptotic proteins such as Puma [27, 28]. As apoptosis is a subsequent event triggered by the DNA damage response pathway to reduce the damaged cell population, DNA DSBs were evaluated via γH2AX staining. The mean number of γH2AX foci per cell increased with dose at doses <88 mGy, and surprisingly declined at doses >88 mGy (Fig. 2). Similarly, Simonsson et al. found the γH2AX foci pattern in the skin biopsies revealed hypersensitivity at <0.3 Gy, but that there was no decrease in foci numbers when the comparison were carried out between the doses of 0.4 Gy and 1.2 Gy at 30 min and 2 h post irradiation [29]. However, the radiohypersensitivity effect is usually demonstrated by the result of the dose relationship for the surviving fraction. Thus, the colony survival assay should be carried

**Fig. 1.** Apoptosis of cells exposed to $2 \times 10^7$ Bq/ml HTO with various doses. Cells were labeled with Annexin V-FITC and PI at 3 h and 24 h post HTO treatment; more than 10 000 cells were collected and the fluorescence was analyzed with flow cytometry. Data were represented as mean ± SD. For comparisons between irradiated groups and sham-irradiated group, values of $P < 0.05$ are referred to as significant.

**Fig. 2.** Average γH2AX foci number per cell produced by $2 \times 10^7$ Bq/ml HTO. Before the cells were fixed, they were washed out with fresh medium twice. Then cells harvested at 3 h or 24 h post radiation were assessed via immunofluorescence. Data are represented as mean ± SD. For comparisons between irradiated groups and sham-irradiated group, values of $P < 0.05$ are referred to as significant.

88 mGy was much higher than that for any other treated group, which was also the case for the level of apoptosis detected 24 h after HTO treatment. Through the period of recovery, the mean foci number produced by the various doses declined to the control level, except for the dose of 88 mGy.
out to confirm whether the radiohypersensitivity effect occurred in hMSCs after acute exposure to HTO. In our work, the remaining foci were down to the control level at 24 h post treatment, suggesting the damage can be efficiently repaired by hMSCs. In contrast to acute exposure, the threat to humans of long-term low-dose-rate radiation is attributed to the bonding of radionuclides with biomolecules under conditions of internal exposure. The concentration of HTO in the medium directly determines the dose rate of the tritium $\beta$-rays; therefore, the influence of the tritium concentration on the cellular response to radiation damage was further investigated. The mean number of $\gamma$H2AX foci per cell produced by different concentrations of HTO depended on the level of radioactivity, which increased with the concentration of the tritium (Fig. 3A). A comparison between the residual foci number measured at 3 h and 24 h later showed that the disappearance rate of $\gamma$H2AX foci in $2 \times 10^6$ Bq/ml HTO was significantly slower than that for $2 \times 10^7$ Bq/ml HTO (Fig. 3B). Similarly, Osipov et al. found that, compared with the significantly decreasing foci after intermediate-dose irradiation (160 mGy and 250 mGy) between 60 min and 240 min post irradiation, a persistence of $\gamma$H2AX foci in hMSCs exists after X-ray radiation with a dose range from 20 to 80 mGy [30]. A threshold level of damage below which cells are strongly impaired in their ability to repair DSBs is demonstrated, and the threshold level corresponds to a dose of $\sim$1 mGy [31]. In our work, the estimated dose after exposure to $2 \times 10^6$ Bq/ml HTO was $\sim$9 mGy, which is above the threshold level of damage in human fibroblast cells. Because of the low linear energy transfer radiation of beta-rays, the persistence of $\gamma$H2AX foci produced by $2 \times 10^6$ Bq/ml HTO is not likely to be related to the complexity of...
DNA DSBs. A possible explanation could be de novo formation of additional foci that are involved in temporary foci occurring in DNA metabolism, such as in DNA replication in the S-phase [32]. The visualized γH2AX foci are the consequence of the phosphorylation of histone H2AX; the activation of phosphorylation is not only related to ATM kinase, but also to the DNA-PKcs and ATR kinases, so this is not just the response to DNA DSBs produced by noxious stimuli, including the ionizing irradiation [33, 34]. The persistence of γH2AX foci in human gingival MSCs produced by low-dose X-rays was found to be ATM independent [30]. ATR is primarily activated in S-phase in response to the collapse of replication forks [35, 36]. Moreover, simultaneous production of free radicals by ionizing radiation at a low dose stimulates MSC proliferation [37], which can ultimately lead to replication stress and result in the generation of secondary DNA DSBs. To investigate such an inference, the cell-cycle distribution was assessed after hMSCs were exposed to a low concentration of HTO (Fig. 4). The proportion of cells in S phase markedly increased, and this was accompanied by a decline in the percentage of cells in G1 and G2 phase. The short-term exposure to a lower concentration of HTO induced hMSC arrested in S phase, which resulted in the persistence of residual foci. As a result, it is reasonable that the number of γH2AX foci in 2 × 10^5 Bq/ml HTO was slightly increased, and had not declined by 24 h post treatment. However, there is reasonable doubt concerning whether the persistence of the foci number was caused by the residual HTO in the irradiated cells, because the time post irradiation in our work was not long enough for the cells to achieve the equilibration of the tritium between the cells and the culture medium. Therefore, the radioactivity of HTO in culture medium incubated with irradiated cells for 24 h was measured every 24 h for 3 days. The radioactivity of the culture medium, due to exchange with the irradiated cells, was ~460 Bq/ml at Day 1, 366 Bq/ml at Day 2 and 346 Bq/ml at Day 3 post irradiation. Further, the damaging effects of kBq/ml HTO were also detected via the cell viability assay and γH2AX foci formation, which sought to analyze the influence of the residual HTO in the irradiated cells. The results showed that the cell proliferation rate after exposure to corresponding radioactivity was similar to that of the sham-irradiated group (65.72 ± 7.48% for the irradiation group versus 72.29 ± 8.18% for the sham-irradiated group, P > 0.05), and the foci number per cell had no marked increase (0.91 ± 0.32 per cell for the irradiation group versus 0.79 ± 0.33 per cell for the sham-irradiated group). Therefore, the residual HTO in the irradiated cells had no obvious impact on the detected γH2AX foci number at 24 h post exposure to 2 × 10^5 Bq/mL or 2 × 10^6 Bq/mL HTO. Liang et al. demonstrated low-dose ionizing radiation by X-rays stimulates the proliferation of rat MSCs, which is associated with the activation of the MAPK/ERK pathway [38]. The MAPK/ERK pathway is reported to be related to numerous cellular behaviors, such as apoptosis, proliferation, differentiation, migration and senescence [38]. Importantly, it has also been shown to play a critical role in the maintenance of self-renewal and tumorigenicity of glioblastoma stem-like cells [39]. Therefore, further work need to be performed to identify the underlying mechanism involved in stem cell response to internal exposure to tritium β-rays, which will help us to understand the role stem cells play in carcinogenesis induced by low-dose ionizing irradiation.

In summary, the biological effects of hMSCs induced by low-dose irradiation from HTO with varying levels of radioactivity were studied in order to help us to understand the impact of radionuclide radioactivity on hMSC behavior after internal exposure. This is useful for estimating the health risk from environmental radionuclides discharged from nuclear power plants and from the clinical application of radionuclide tracing techniques in diagnosis. After acute exposure to 2 × 10^5 Bq/ml HTO, a slight increase in apoptosis was detected in hMSCs, except for at the dose of 9 mGy. It was found that the γH2AX foci can be efficiently repaired by hMSCs. Moreover, a shift in the cell cycle indicated lower-dose-radioactivity HTO induced cell arrest in S phase, which resulted in temporary...
foci formation occurring during DNA replication. This contributed to the persistence of γH2AX foci when hMSCs were exposed to lower concentrations of HTO.

CONFLICT OF INTEREST
The authors report that there are no conflicts of interest.

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