The response of mouse embryonic stem cells to low doses of γ-radiation: Evidence for an adaptive response

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1. Introduction

The responses of organisms to low dose/low dose rate ionizing radiation have attracted great attention. The “hormesis” model, which proposes that the dose–response curve is bell-shaped [1], challenges the conventional view that dose responses are monotonic [2]. It is consistent with an “adaptive response” (AR), in which, prior exposure to a low dose of an agent, protects against later exposure to a high dose. AR has been studied in various somatic cell systems [3–12]. However, in an animal, such as a mouse, stem cells, rather than somatic cells, determine the response to radiation [13]. Differences in the occurrence and amplitude of defensive effects such as AR are observed in among specific cells, tissues, individuals, and animal species and individuals, it is speculated that there is a genetic and physiological basis for AR in cells [14]. The focus of this study is to test whether AR can be induced in mESCs or restricted to specific cell types. For this purpose, the stem cell line Royan-B1 was used. In 1990, an AR was reported in C57BL/6 mice, pre-exposed to low doses of ionizing radiation [5]. Because of the determinative role of stem cells in organism sensitivity [13], stem cells derived from the C57BL/6 mouse are also expected to be resistant to ionizing radiation. Cell line RB-1, available in our center, is derived from this strain [15].

In the present work, we demonstrate, using the cytokinesis-blocked micronuclei assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (MTT), the induction of AR in mESCs after exposure to a low dose of γ-radiation.

2. Materials and methods

2.1. Cell culture

RB-1 cells were cultured in Knockout-Dulbecco’s modified Eagle medium (KO-DMEM) (10829-018; Invitrogen, USA) supplemented with 15% ES-qualified fetal bovine serum (ES-FBS) (10439-024; Invitrogen, USA), 2 mM L-glutamine (15039-027; Invitrogen, USA), 0.1 mM β-mercaptoethanol (M7522; Sigma–Aldrich, Germany), 1% nonessential amino acids (11140-035, Invitrogen, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (15070-063; Invitrogen, USA). For 50 μl KO-DMEM, 50 μl leukemia Inhibitory Factor (LIF) (ESGROTM; 13275) was added. Cell cultures of mESCs were established as previously described [16].

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2.2. Pre-treatment chromosome analysis

Prior to irradiation, cells with same passage numbers were analyzed for possible chromosomal abnormalities. Cytogenetic evaluation was carried out based on a standard protocol [17]. Metaphase spreads with 40 chromosomes and no chromosomal aberrations were considered to be normal.

2.3. Irradiation with γ-rays

Typically, the AR is induced with 1–100 mGy of γ-rays [18]. In this study, the G1/S phase synchronized, Matrigel® coated mESCs were prepared [19] in a T-25 tissue culture flask (90025; TPP, Switzerland) and irradiated with 40Co γ-rays (Theratron II 780-C, AECL, Kanata, Ontario, Canada) with dose rate 130–136 mGy/min. Cells were exposed to 2.5, 3.7 and 5 Gy γ-rays as adaptive dose (D1), with or without a challenge dose. 2.5 Gy was the lowest possible dose for the irradiator. The challenge dose (D2) was 150 Gy. The interval between D1 and D2 exposures was 5 h. The sham group was not irradiated. The control group was exposed only to the challenge dose (D2). In total, for each experiment (series), 11 discrete groups were irradiated. Three independent experiments were carried out.

2.4. Micronucleus assay

In this study, on the basis of standard methods, most of the mESC population underwent DNA fragmentation after treatment with cyt-B. As the cytotoxicity of cyt-B varies between cell types, and even between subtypes of the same cell type [20], we modified the conventional MN assay for mESCs [19]. After different trials, seeding the mESCs on feeder cell layers and then covering them with Matrigel for 24 h turned out to be the best culture condition for these cells.

Immediately after D2, for observation of the stimulating effect of low-dose radiation (LDR), cells were treated with cyt-B at final concentration 4 μg/ml (c-6762; Sigma–Aldrich, USA) and placed in an incubator (37 °C, 5% CO2) for 8 h. Afterwards, the cells were trypsinized and spun down at 800 g for 6 min. Hypotonic solution (0.6% KCl, 4 mL) was added, to obtain good preservation of the cytoplasm. After keeping the cells at room temperature (RT) for 10 min, cool fixing solution (3:1 methanol:acetic acid, 0.5 ml) was added dropwise as pre-fix, followed by spinning down the cells at 800 g for 6 min. The cell pellet was gently fixed with cool fixative for 15 min at RT and the fixative was changed twice. Finally, the suspension was dropped on wet ethanol–cleaned slides. The air-dried slides were stained with Hoechst 33258 (382061; Calbiochem, UK), and examined under a fluorescence microscope (Olympus BX51, Japan) equipped with a filter for DAPI (blue) (Fig. 1). MN were scored according to criteria describe by Fenech [21]. Cells were scored in order to determine MN frequency. At least 1000 bi-nucleated cells per slide were evaluated for each dose in each experiment. The frequency was expressed as number of MN per bi-nucleated cell (mean ± SD). The frequencies of cells with one, two or three MN were recorded.

2.5. MTT assay

In order to determine whether the lowest dose could modulate cellular response of mESCs to a subsequent high dose of radiation, further studies were conducted. Cell viability was assessed using the MTT assay [22]. Following 2 h incubation after exposure to D2, the cells were harvested and 5 × 10^4 cells were cultured in each well of a 96-well tissue culture plate while incubated with MTT solution (0.5 mg/ml) in fresh medium at 37 °C for 2 h. After removing the supernatant, dimethylsulfoxide (DMSO) was added to each well of the cell culture plate, followed by mixing with a pipette until the formazan crystals were completely dissolved. The absorbance of formazan was measured at 570 nm using standard microplate absorbance readers (ELISA reader Biotek-ELX 800). In all experiments, eight replicate wells were used at each point.

2.6. Mycoplasma detection

It is becoming increasingly evident that masked mycoplasma contaminations can inhibit the growth and metabolism of cultured cells [16]. Since induction of AR is completely dependent on cell metabolism [23,24], in our experiments, we verified the absence of mycoplasma contamination, using DNA staining [25,26].

2.7. Statistical analysis

Data from treated and untreated (used as sham group) cells were expressed as mean ± SD from three independent experiments. In order to assess whether the means of two groups of samples are statistically different, a t-test was carried out with SPSS software (version 13). Microsoft Excel 2010 was used to draw the graphs. A p < 0.05 was considered to be statistically significant.

3. Results

To determine the optimal low D1 that could induce an AR in mESCs, cells were exposed to 2.5, 3.7, or 5 Gy γ-rays in hypoxic environment with 150 Gy, and the MN frequencies were measured. As shown in Fig. 2, MN increased in a dose-dependent manner. The level of MN in the group which received D1 = 2.5 Gy before exposure to D2 was lower than in the group which received D2 only (0.164 vs. 0.24; p < 0.05). This may indicate a protective effect of D1. Other pretreatment regimens were not protective against MN induction and a decrease in survival was also observed (p > 0.05).

In the most recent revision of ISO 10993 (Biological Evaluation of Medical Devices), the MTT assay was prescribed as a quantitative cytotoxicity technique which has the potency to be used as an indicator for the AR evaluation [27]. The MTT results showed, exposure to D2 alone, reduced absorbance from 189 ± 0.06 in the untreated group to 144 ± 0.04. Cells exposed to D1 only showed values of 180 ± 0.05, 172 ± 0.13, and 163 ± 0.14 (2.5, 3.7, and 5 Gy, respectively); Fig. 3. In cells receiving D1 + D2, absorbance were 170 ± 0.04, 135 ± 0.06 and 115 ± 0.23 (D1 = 2.5, 3.7, and 5 Gy, respectively). We conclude that cells irradiated at D1 = 2.5 Gy show an AR.

4. Discussion

In accord with the Bergonie–Trébousteau law, the number of undifferentiated cells in the tissue, their mitotic activity, and the length of time that they are actively proliferating [28], are the major factors which determine the radiation sensitivity of a given tissue; on this basis, stem cells would be more vulnerable to radiation. Hence, stem cell sensitivity may be determinative of the overall sensitivity of a biological system [13]. Moreover, in tissues, it is generally assumed that cell cycle regulation is the most important determinant of a cell’s sensitivity to ionizing radiation. On the other hand, because of the self-renewal capacity of mESCs, they are mostly in the S-phase of the cell cycle, which is the phase least sensitive to radiation [29]. Furthermore, there is controversy regarding the adaptability of undifferentiated vs. differentiated cells. Some workers asserted that undifferentiated cells (e.g. neoplastic tissues) exhibited far greater adaptation than normal cells; others thought that low-dose radiation induces AR in normal cells but not in neoplastic cells [30,31]. Because of all the above reasons, stem cells vs. somatic cells may be more appropriate for better understanding the effect of low radiation doses in living organisms.

We used the CBMN assay, since AR could be seen after low-dose ionizing radiation at the level of chromosomal aberrations.
This assay is a multi-endpoint test [32] which detects aneugens and clastogens. In addition to the simplicity of scoring, validation by different authors, wide-ranging applicability in different cell types, and predictive value for cancer [33], it has more statistical power than the chromosomal aberration test [34,35]. This technique has been used for lymphocytes, fibroblasts, and epithelial cells, but not yet widely used with embryonic stem cells.

Until now, AR has been investigated mainly in differentiated cell systems. Here, we observed that a primary dose D1 = 2.5 cGy may be protective, when followed by a later high dose (Fig. 2).

Following exposure to ionizing radiation, possible cell fates include necrosis, apoptosis, interphase death, and biological adaptation [36]. Our data are consistent with a model in which, in the initial period after irradiation, necrotic cells are the only group of cells that lost their ability to reduce MTT. Hence, choosing an appropriate time following D2 for interpretation MTT assay results may be crucial for analyzing AR. Sawada et al. found that the best time for analysis of apoptosis was 16 h post-treatment [37]. Apoptosis is considered to be “active” cell death; early-stage apoptotic cells may retain metabolic activity to reduce MTT. So, an increase in MTT reduction is not equal to additional survival of cells. With extending the incubation time, early-stage apoptotic cells ultimately undergo “secondary necrosis”, which eventually halts all metabolisms. These cells are gradually eliminated from the population, so this may be the best time at which to measure metabolism. There is a direct relationship between the AR to ionizing radiation and the level of MTT reduction. Therefore, the adapted group was expected to have the highest MTT reduction rate (Fig. 3).

5. Conclusions

Despite the suggestion that stem cell biology has “come of age” [38], therapeutic applications of embryonic stem cells may still be far from clinical realization. We have found that there is a low-dose radiation window for initiation of the AR in mESCs. The finding that mESCs can show an AR may stimulate research on other types of stem cells. There is an ongoing discussion on the radio-resistance of cancer stem cells [39,40], but the resistance of normal stem cells also merits further study [41].

Conflict of interest statement

The authors have no conflict of interest to declare.

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