

COMET ASSAY STUDY OF DNA BREAKAGE AND APOPTOSIS IN MICE EXPOSED TO LOW DOSE-RATE IONIZING RADIATION

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The DNA strand breaks level and apoptotic cells rate in spleen cells of CBA male mice continuously (40-365 days) exposed to very low dose-rate (0.17 cGy/day) γ -radiation were investigated using the comet assay. It was shown that very low dose-rate irradiation resulted in statistically significant increase in the DNA strand breaks level, starting from a dose of 20 cGy. Further prolongation of exposure time and, hence, increase of a total dose did not, however, lead to further increase in the DNA strand breaks level. At the doses 20, 45, and 61 cGy approximately two-fold increase over a control level in the apoptotic cell fraction was observed.

Keywords: *ionizing radiation, very low dose-rate irradiation, DNA strand breaks, apoptosis, comet assay.*

INTRODUCTION

All living organisms, including human, are continuously exposed to ionizing radiation (IR) from natural sources. However, development of nuclear technologies and associated intentional (e.g., Hiroshima and Nagasaki) and accidental (e.g., Chernobyl) releases of radioisotopes have led to increase in a background level of IR. This technogenic part of IR exposure has risen significantly over last few decades.

While there are no doubts about negative biological effects of high dose IR, debates about whether low dose IR exposure is harmful or beneficial (hormetic) are still continuing among scientific community. Analysis of available literature [1,2] show that low dose IR exposure induces a complex of biochemical and biophysical reactions in animals. It is not clear, however, whether those changes are consequences of organism adaptation to increase in IR background, and whether low doses cause any significant genetic alterations.

The aim of the present work was to study of DNA strand breaks and apoptosis induction in spleen cells of CBA male mice continuously (40-365 days) exposed to very low dose-rate γ -radiation at a dose rate of 0.17 cGy/day.

MATERIALS AND METHODS

4-5 week old CBA/lac male mice weighting 12-14 g (purchased from "pitomnik-Stolbovaya") were used in the experiments. The mice were placed in plastic cages 7-14 days prior to IR exposure. Distribution of animals into control and experimental groups was random.

Mice were given standard dry feed and water *ad libitum*. Experiments with chronic low dose-rate irradiation were carried out from 2000 to 2005.

The experimental animals were chronically exposed to IR from a gamma-ray unit "UOG-1" (VNIIFTRI, Russia) equipped with a cesium-137 source (activity 7.2×10^8 Bq) mounted in a steel container and specifically designed for long-term irradiation of biological objects. The IR source was placed above exposed targets. Chronic irradiation of animals was performed at a dose rate of 0.07 mGy/h (distance from mouse bedding to the γ -radiation source was 64 cm, filter-lens #5). Variability of a dose-rate within area to be irradiated (1 m^2) did not increase 10%.

Irradiation was continuous with 10-15 min daily break for hygiene procedures. Control dosimetry was performed using thermoluminescent detectors TLD-100 (Sweden) and DTG-4 (Russia). At 40, 80, 120, 270, and 365 days of exposure, mice were sacrificed and spleen and red bone marrow were removed and processed for subsequent analysis. Total cumulative doses delivered to mice were 7-61 cGy, respectively.

Suspension of spleen cells in phosphate buffered saline (pH 7.4) containing 0.14 M NaCl, 2.7 mM KCl, 3 mM NaN_3 , was filtered through nylon mesh at 4°C. Cell concentrations were counted using hemocytometer.

Alkali single cell gel electrophoresis was carried out as described by Singh et al. [3]. According to the assay, a number of alkali labile sites and single-strand breaks is proportional to a number of DNA fragments and to distance DNA migrated from the nucleus after alkali electrophoresis of agarose-immobilized single cells. Fluorescent dye Hoechst 33258 (Sigma Chemical Co, St. Louis, MO, USA) was used to visualize DNA. Analysis was performed using the "Lumam I-2" fluorescent microscope (LOMO, Russia). 100 comets were counted from each slide. Comets were divided into classes 0-4 (0 corresponded to no visible tail, 4 – total migration of DNA from the nucleus into the tail) depending on the shape (diameter, tail length, etc.). This method of visual damage is considered as a valid way for DNA damage analysis [4]. The results of the visual classification were subsequently confirmed using the analytic package image analysis software (Kinetic Imaging, Liverpool, UK).

Numbers of comets in each class were scored and the average comet index (ACI) was calculated as: $ACI = (1 \cdot n_1 + 2 \cdot n_2 + 3 \cdot n_3 + 4 \cdot n_4) / \Sigma$

$n_1 - n_4$ – a number of comets in classes 1–4, и Σ – the sum of counted comets, including comets in class 0.

Percentage of apoptotic cells was determined by the DNA diffusion modification of the comet assay described elsewhere [5].

Statistical analysis of experimental results was performed using the Student t-test. Results are presented as averages for a dose group of animals \pm standard errors.

RESULTS AND DISCUSSION

Figure 1. shows the results of ACI determination in spleen lymphocytes from mice exposed to chronic very low dose-rate IR. In the range of about 7 to 20 cGy (40-120 days), a linear increase in ACI coefficient occurred. Further accumulation of a dose does not, however, lead to further increase in ACI. Its value stayed at about the same level in the range of 20 to 61 cGy.

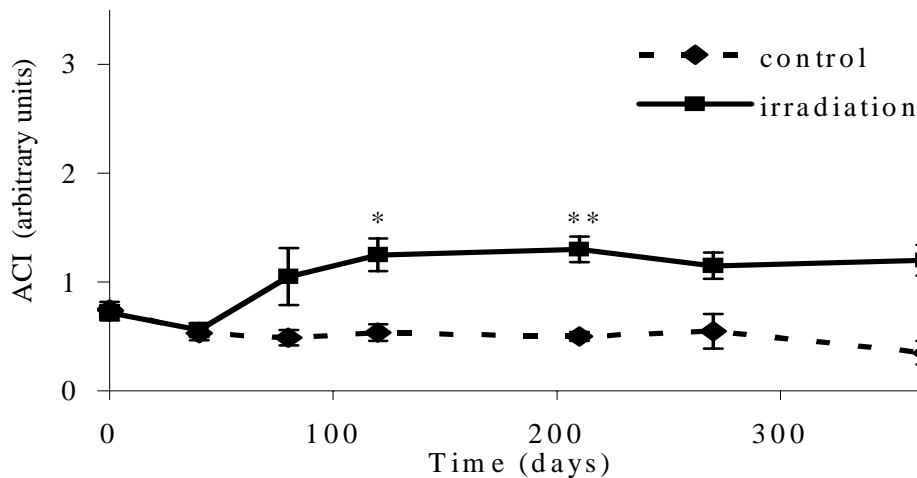


Figure 1. Chronic γ -irradiation induced time-dependent changes in the average comet index (ACI) in mouse spleen lymphocytes. * $p < 0.05$, ** $p < 0.01$

It is well known that some background level of DNA breaks is always present in mammalian cells due to normal metabolism. These breaks can be divided into two classes:

- breaks resulted from DNA damage by free radicals produced by cellular oxygen metabolism;
- breaks resulted from DNA replication, repair, transcription processes, as well as chromatin condensation and decondensation.

An IR dose of 1 Gy induces approximately 1000 DNA single strand breaks per cell [6]. Simple calculations based on these data show that irradiation with a dose-rate of 0.17 cGy/day will induce about 2 DNA single strand breaks per cell per day, whereas a number of endogenous single strand breaks resulted from normal oxygen metabolism is $\sim 1.2 \times 10^5$ per cell per day [6]. It is, therefore, obvious that low-dose rate IR-induced DNA SSBs can hardly influence a total number of DNA breaks. Nature of spontaneous and induced DNA breaks is rather similar. IR-induced primary lesions could lead to genotoxic effects only in case if spatial distribution of the lesions along the chromatin and effectiveness of their repair is different from those spontaneously induced. Studies of last decade suggest that the particular areas within chromatin possessing hypersensitivity to IR do exist [7]. Double-stranded DNA clusters composed of multiple lesions on opposing DNA strands within a few helical turns are of particular danger to cells, since it is hard for DNA repair machinery to repair them [8]. These clusters are thought to be crucial IR-induced DNA lesions leading to DSBs, and eventually to mutagenesis and cell death [9]. Sutherland et al. [10] demonstrated that as low as 10 cGy IR caused an increase in clustered DNA damage level in human monocytes. Consistent with our results, non-dividing primary human fibroblasts exposed to 1 mGy of IR were not able to repair DNA DSBs for several days, while effectiveness of DSB repair after higher doses was much better [11].

A minor part of cells that has an extremely high level of DNA damage (e.g., apoptotic cells) would supposedly contribute substantially to an overall DNA damage level within an entire cellular population. To take into account the contribution of an apoptotic cell subpopulation to a final readout of DNA breaks in our experiments, we measured the percentage of apoptotic spleen lymphocytes from mice exposed to very low dose-rate IR or untreated animals using the "DNA diffusion" assay. At the days 120, 270, and 365 of the chronic irradiation (20, 45, and 61 cGy,

respectively), approximately two-fold increase over a control level in the apoptotic cell fraction was observed (Table. 1).

Table 1. Percent of apoptotic spleen lymphocytes in control mice and mice exposed to very low dose-rate IR.

Length of experiment (days)		40	80	120	210	270	365
Apoptotic cells (%)	Control	1.1±0.9	1.2±0.8	1.8±0.4	0.8±0.7	2.0±0.6	1.6±0.7
	IR	1.7±0.8	2.2±1.0	3.6±0.6*	2.9±0.8	4.1±0.7*	4.8±1.1*

* Significantly increased compared to control ($p < 0.05$).

As expected, a correlation ($r=0.86$; $P<0.05$) between an overall level of DNA damage and percentage of apoptotic cells was noticed. These observations prompted us to recalculate overall DNA damage levels (the *ACI* coefficient) in irradiated vs. untreated groups. When performed without counting highly damaged cells (comets within classes 3 and 4), the comet assay yielded in less, but still statistically significant, difference in DNA damage levels between irradiated (20-61 cGy) and untreated mice (data not shown). The appearance of apoptotic cells with highly fragmented DNA in irradiated animals cannot, therefore, have direct impact on overall DNA strand breaks level. An indirect mechanism, also known as a «bystander effect», response of unirradiated directly cells due to signaling originating from irradiated cells [12], can be possibly involved in the effects observed in this study. In this scenario, lesions within supersensitive chromatin regions in a minor, apoptotic cell population, appear to trigger a cascade of metabolic processes in different cell populations on both organ and organism levels. Bystander effects have been demonstrated after both low-LET and high-LET IR exposures [13]. A signal from irradiated cells can be transmitted by direct intercellular contacts (gap junction communications), as well as by cytokines and/or oxygen species secreted by irradiated cells [14]. A variety of changes has been reported to occur in bystander cells, including overproduction of free radicals [15,16], induction of stress-related kinases JNK, ERK1/2, and others [17], cytokines Beta1-integrin and IL-1alpha [18]. In addition, reactive oxygen species (ROS) can act as signal molecules to propagate and regulate a particular cellular response, such as proliferation, differentiation, and apoptosis [19]. It is well known that actively transcribed DNA sequences are much more susceptible to DNA damage than those in compact chromatin regions due to unlimited accessibility of them for reactive oxygen species (ROS) [20,21]. Increase in actively transcribed genes, together with an increase in ROS production can, therefore, lead to elevated DNA damage. On the other hand, DNA damage within active genes is repaired faster and more efficiently compared to that in silent genes [22, 23].

Our hypothesis is supported by results of monitoring reparative and replicative DNA syntheses in mouse bone marrow cells reported by Mazurik et al. [24], within a collaborative effort with our group, performed on the same mice used in our present study. The authors demonstrated that chronic low dose-rate irradiation of mice substantially induced reparative and replicative DNA syntheses in bone marrow cells (60% and 67% increase; $P<0.01$, $P<0.01$, respectively). As mentioned above, activation of DNA replication and repair is associated with increase in DNA strand breaks level. Besides, significant positive correlation ($r = 0.87$; $p<0.01$) between DNA strand breaks and superoxide anion-radical content in bone marrow cells of the irradiated mice was shown, indicating additional production of DNA damage by ROS due to the loss of a part of structural proteins and conformational changes in expression sites of the chromatin during gene expression [24].

It was suggested that the bystander effect has an alternative, protective, nature by means elimination of highly damaged, potentially dangerous cells from a cell population. [25,26]. In accordance with this line of evidence, an adaptive response was associated with overproduction of reactive oxygen species [19]. In our previous study we demonstrated that increase in DNA breaks level by the days 120-365 of low-level IR exposure is accompanied by elevated radioresistance to hydrogen peroxide treatment [27]. It was suggested that the elevated cell radioresistance can be explained by either activation of DNA repair, or elimination of a supersensitive cell population [27].

To summary, chronic irradiation at a dose-rate 61 cGy/year lead to statistically significant increase in a number of DNA strand breaks within a 20-61 cGy dose range. Overall increase in the level of DNA breaks in mouse spleen cells as a result of chronic low dose-rate IR exposure can be associated with the chromatin rearrangement accompanied by gene overexpression, increase in ROS production rate, and DNA repair activation, processes known to be triggered after low doses of IR. Although insignificant, a contribution of apoptotic cells to an overall level of DNA damage was also recorded.

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