J. Radiat. Res., 47, 279–285 (2006)

Regular Paper

Adaptive Response of Blood Lymphocytes of Inhabitants Residing in High Background Radiation Areas of Ramsar- Micronuclei, Apoptosis and Comet Assays

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High Natural Radiation Areas in Ramsar/Micronuclei/Apoptosis/Comet assay/Radio-adaptive Response.

The hot springs in certain areas of Ramsar contain ^{226}Ra and ^{222}Rn. The effects of natural radiation on the inhabitants of these areas and the inhabitant's radiosensitivity or adaptive responses were studied. One group of volunteers from areas with high natural background radiation and another group from areas with normal background radiation were chosen as the case and control group respectively. The frequency of micronuclei, apoptosis, and DNA damage in peripheral blood mononuclear cells were measured following γ irradiation (4 Gy). The incidence of micronuclei in the case group was significantly lower than that in the control group while their frequency of apoptosis was higher ($P < 0.05$). However, the rates of induced DNA damage and repair were significantly higher in the case group ($P < 0.05$). Smaller number of micronuclei and higher levels of apoptosis in the case group could be the result of higher resistance to radiation stress and a more rigorous checkpoint at cell division. However, regarding the alkaline labile sites, the individuals in the case group are more sensitive and susceptible to DNA damage. The results of micronuclei, apoptosis and repair studies suggest that an adaptive response might be induced in people residing in areas with high background radiation.

INTRODUCTION

Human biomonitoring, as a tool for identifying health risk from environmental exposures, has gained increasing interest especially in the areas of cancer risk assessment and response to therapy.[1–3] A major objective of these studies is to provide a direct assessment of the risk of exposure to ionizing radiation at low doses and low dose rates, including evaluation of the adequacy of risk estimates that serve as the basis for radiation protection standards. Natural radionuclides in soil generate an important component of the background radiation exposure of the population.[4–7] Epidemiological and radiobiological studies in high natural background radiation areas (HBRAs) have provided the opportunity to directly observe the effects of long-term radiation on human beings. In several high background radiation areas of the world, such as Brazil, India, China and Iran, the radiation levels of the local inhabitants are similar to or above those of people employed in the nuclear industries or radiation medicine.[8–12] Among above mentioned countries, Ramsar located in Iran, has attracted special attention. Due to the presence of natural radionuclides, especially ^{226}Ra in hot springs, the inhabitants in some areas of Ramsar receive an annual effective dose of up to 260 mSv/y. This effective dose is substantially higher than the 20 mSv/y, which is permitted for radiation workers.[13] However, a number of studies on HBRAs of Yangjiang in China have not shown any evidence of increased cancer mortality.[14–15] In fact, data on the effects of high-level natural radiation on some cytogenetic parameters in the inhabitants of HBRAs are very much limited. In some of these studies,[16–19] the incidence of chromosomal aberrations in HBRAs was lower than that in normal back ground radiation areas (NBRAs). In another cytogenetic analysis, higher incidences of stable and unstable chromosomal aberrations were observed in the residents of HBRAs compared with the control group.[20] There are different reports on the effects of chronic low-level irradiation; while an adaptive response is observed in some cases,[21–24] clastogenic effects are observed in others.[23,25]

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doi:10.1269/jrr.0575

DNA is considered to be the primary target during the destruction of cells by ionizing radiation. Gamma irradiation is able to break DNA through the deposition of energy in the deoxyribose phosphate backbone.[26–28] A wide variety of methods have been used for the detection of early biological effects of DNA-damaging agents in environmental and occupational settings. These include well-established biomarkers such as chromosome damage measured by chromosome aberration (CA) and sister chromatid exchange (SCE). However, both methods are laborious and time consuming. The complexity and laboriousness of enumerating aberrations in metaphases have stimulated the development of a simpler system for measuring chromosomal damage. Schmid[29] and Heddle[30] proposed independently that measurement of micronuclei was an alternative and simpler approach to assess chromosomal damage *in vivo*. Micronuclei and apoptotic cell death are biological indicators for the assessment of radiosensitivity.[31–33] In addition, comet assay has shown to be both sensitive and rapid in the detection of breaks in the DNA-strands of individual cells.[34–37]

In the present study, various biological methods (micronuclei, apoptosis and alkaline comet assays) were used to evaluate the induction of the adaptive response to low dose ionizing radiation in residents of HBRAs.

MATERIALS AND METHODS

Study populations and sampling

The present study was carried out in Ramsar. As the case group, thirteen healthy individuals belonging to the HBRAs of Ramsar (17–61 years old) were chosen (from Talesh Mahaleh, Chaparsar, and Abe-siah quarters). All individuals filled in a detailed questionnaire covering their occupational, medical and family history, habitual behavior of food consumption, drug usage, smoking tobacco and drinking alcohol, etc. Radiation doses at 5 or 6 corners of each house were measured directly using a Multi-Purpose detector (Rados RDS-110, RADOS Technology, GmbH Hamburg, Germany) and the average value was calculated as the indoor dose. As a control group, fourteen healthy volunteers (17–59 years old) who were matched for age, sex and smoking habits were chosen from NBRAs in Ramsar.

A 10 ml aliquot of heparinized peripheral blood was collected via venipuncture from each volunteer with their full informed consent. For apoptosis and comet assays peripheral blood mononuclear cells (PMNCs) were isolated from the heparinized blood samples by centrifugation at 800 × g for 30 min at 20°C on Lymphoprep (AXIS – SHIELD PoC As) then resuspended in RPMI 1640 (Gibbco BRL) with 10% fetal calf serum (Sigma). For the micronuclei assay, whole blood was diluted in L-glutamine-modified RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (Gibbco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin (Boehringer Mannheim).

Irradiation

Each sample was divided into two parts; one part was irradiated with 4 Gy ^{60}Co γ rays (Gamma Beam 150, Atomic Energy Canada) at a dose rate of 0.5 Gy/min. In order to inhibit DNA repair, the cells were incubated in cold ice. The other part was unirradiated and incubated in cold ice.

Micronuclei assay

The irradiated samples were incubated at 37°C for 1 hour in a humidified atmosphere containing 5% CO_2. Afterwards, to stimulate lymphocyte proliferation, phytohemagglutinin-M (Boehringer Mannheim) was added at a concentration of 15 μg/ml. After 44 hours of incubation at 37°C, 4.0 μg/ml cytochalasin B (Sigma) was added, and the cells were harvested after 72 hours of incubation. The cells were treated with a hypotonic solution containing 0.075 M KCl for 4–6 minutes. Following centrifugation at 4500 × g for 5 minutes, the cells were fixed in a 3:1 mixture of methanol and acetic acid. The cells were then dropped onto cooled slides and air-dried. The slides were coded, stained in 10% Giemsa (Merck), and the micronuclei were scored in 1000 binucleate cells under the microscope at a magnification of 400 × based on the criteria of Fenech.[38]

Apoptosis assay

The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 for 48 hours. The cultures were harvested by centrifugation at 4500 × g for 5 minutes. This was followed by keeping the resulting cell pellets in a fixative containing a mixture of 3:1 methanol: acetic acid for 10 minutes. The cells in the resulting suspension were collected by centrifugation (4500 × g for 5 minutes), and the fixative was removed. The resulting cell pellets were then resuspended in 0.5 ml of the fixative, and approximately 50 μl of this suspension was spread onto a microscopic slide. The slides were air-dried, coded, stained in 10% Giemsa and examined under a microscope at a magnification of 1000 × . For each case, the apoptotic cells were scored in 1000 cells.

Comet Assay

Slide preparation: Immediately after irradiation, an aliquot of the cell suspension was processed to evaluate the initial damage. DNA strand rejoining was allowed to proceed by incubating another aliquot of treated cells for 2 hours in RPMI 1640 with 10% fetal calf serum in the presence of Penicillin/Streptomycin 100U/100 μgr/ml, at 37°C in 95% humidified air and 5% CO_2. Alkaline comet assay was performed according to Singh *et al*.[39] Slide preparation of the sandwich type was carried out by embedding 40000 lymphocytes. The slides were transferred into a chilled lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10mM Tris, 10% DMSO and 1% Triton X-100, pH 10) and incubated at 4°C in the reduced light for 60 minutes. They were then placed into a horizontal gel electrophoresis tank and covered with

fresh buffer (1mM Na_2EDTA, 300mM NaOH, pH> 13), for 20 minutes, and then electrophoresed at 0.56 V/cm and ~290mA for another 20 minutes. Finally, they were rinsed with neutralization buffer (0.4M Tris, pH 7.5) for 15 minutes, fixed by methanol and stained with 20 μg/ml ethidium bromide.

Evaluation of DNA damage: The slides were coded and examined at 200x magnification using a fluorescence microscope (Nikon Eclipse E600). For each case, between 100–150 cells were analyzed and the results were normalized to 100. The extent of DNA migration was evaluated according to the criteria established by Anderson *et al.*[40] The comets were classified into five categories (0–4) according to the extent of DNA damage. Comets with a bright head and no tail were classified as class 0 and comets with a small head and a long diffuse tail, as class 4, i.e. highly damaged cells. Comets with intermediate characteristics were classified as classes 1, 2 or 3. The extent of DNA damage (DD) was quantified by the formula described by Jaloszynski *et al.*[41]:

$$DD = (n_1 + 2n_2 + 3n_3 + 4n_4)/ \Sigma \quad (1)$$

where DD is the DNA damage in arbitrary units (au), n_1–n_4 are the number of comets in the classes of 1–4, and Σ the total number of scored comets, including class 0.

The amount of repaired DNA was estimated using the following formula:

$$R = (DD_{0h}–DD_{2h})/ 100 \quad (2)$$

where R represents the repairing of DNA damage, and DD_{0h} and DD_{2h} represent DNA damage at 0 and 2h after being irradiated with 4 Gy of radiation.

Statistical analyses

Data were analyzed using the statistical analysis system software packages of Microcal Origin and Excel. Analysis of Student's t-test with two- sample unequal variance was used to assess the differences between experimental groups, and the level of signification was set as 5%.

RESULTS

To elucidate the effects of radiation on lymphocytes in individuals of HBRAs and NBRAs, the incidence of micronuclei, induction of apoptosis and the amount of initial and residual damage after irradiation to the challenging dose of 4 Gy of γ ray were examined, which are presented below.

Figure 1 depicts the micronuclei frequencies in 1000 binucleate lymphocytes belonging to both groups, before and after exposure to the challenging dose. The spontaneous levels of micronuclei in the binucleate cells for the case group were not significantly different from the control group. Radiation induced significantly less micronuclei in the case group than the control group. This result clearly indicates that priming irradiation with low chronic doses in the HBRAs rendered the cells less susceptible to the challenging dose.

Figure 2 presents the results of the apoptosis assay in both groups, at 0 and 4 Gy of the challenging dose followed by 48 hours of incubation at 37°C. In the non-irradiated samples, as in the micronuclei assay, the induction of apoptotic cells in the two groups was not significantly different. However, after exposure to the challenging dose, the apoptosis level in the case group was significantly higher than that in the control group. This result suggests that either a strict checkpoint system is present or more damage is induced by irradiation in the case group.

These observations prompted us to examine the induction of DNA damage and its repair by means of the comet assay.

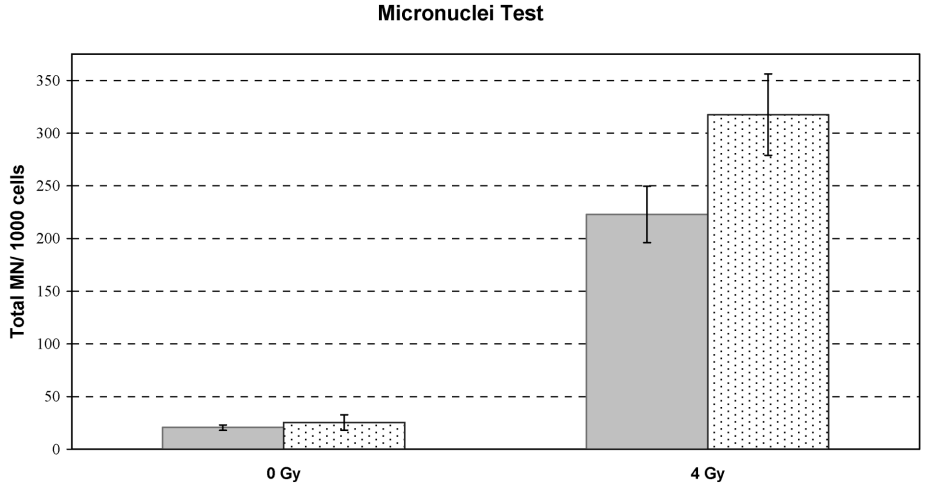

Fig. 1. Micronuclei frequencies in 1000 binucleate lymphocytes for the case (solid bars) and the control group (dotted bars). Micronuclei occurrence for the case group was significantly lower than the control group ($P < 0.05$) after exposure to 4 Gy of the challenging dose of γ-rays.

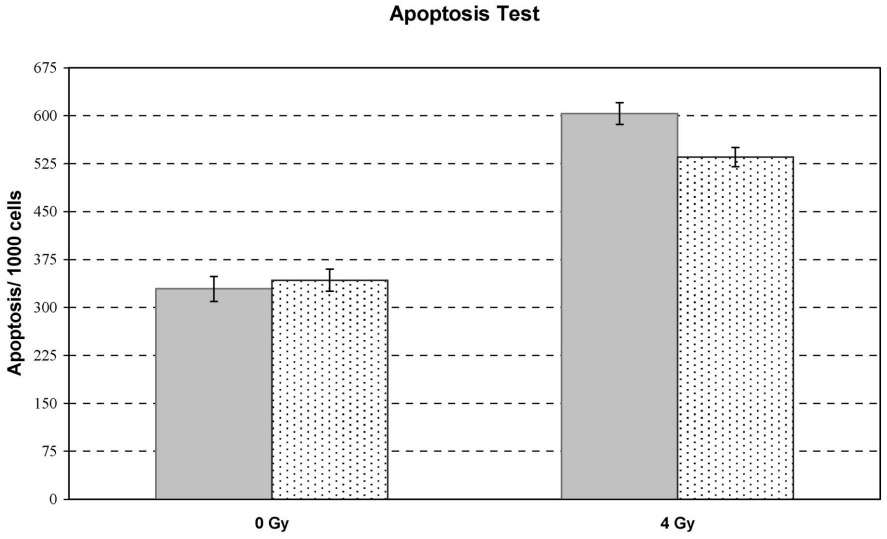

Fig. 2. Spontaneous and the induction of apoptotic cells after exposure to the challenging dose in the case (solid bars) and the control group (dotted bars).

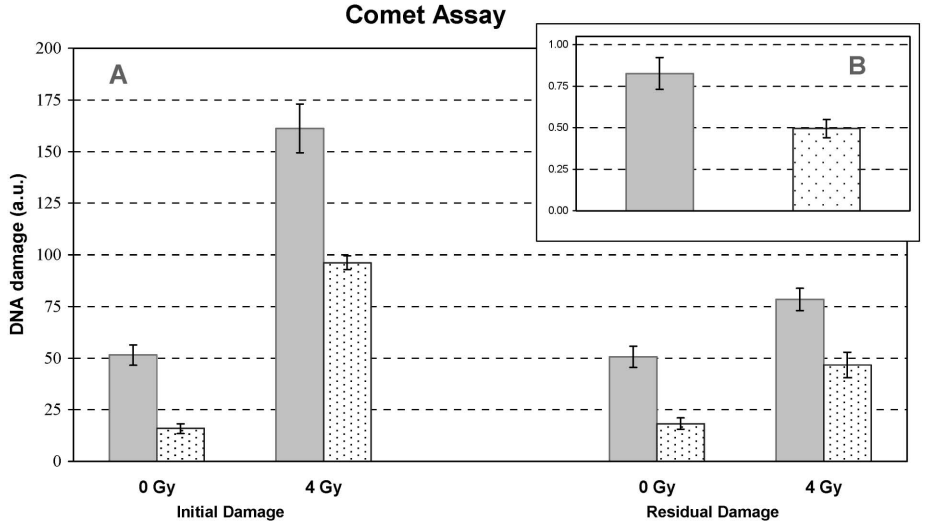

Fig. 3. The initial and residual DNA damage (A) and the amount of repaired DNA (B) of lymphocytes measured by comet assay in the case (solid bars) and the control group (dotted bars). In the case group, the initial damage was higher ($p < 0.05$) than the control group. The amount of DNA repair was calculated by formula 2 and underscores the improved repair ability within the case group.

The results of initial and residual DNA damage in lymphocytes for both groups are presented in Fig. 3-A. It is remarkable that the spontaneous level of DNA damage in the case group is significantly higher than that of the control group. This could mean that residual damage results mostly from chronic low-level irradiation and/or more alkaline labile sites are present in this group. After introducing a challenging dose, more damage is induced in the case group ($p < 0.05$) indicating more susceptible sites to break and/or more alkaline labile sites are present in lymphocytes of the case group. However, after two hours of incubation, the major parts of the damage were repaired (Fig. 3-A). Figure 3-B represents the amount of repaired DNA in the case and control groups, calculated according to formula (2), and underscores the improved repair ability within the case group.

DISCUSSION

In this study the effects of chronic low doses of radiation in human lymphocytes were evaluated among inhabitants in HBRAs. Radiation can induce DNA damage in cells and it has been shown that unrepaired or misrepaired DNA breaks can lead to apoptotic death or chromosomal damage.[42] It is generally assumed that cellular radio sensitivity is indicated

by radiation-induced micronuclei and/or apoptosis.[43] Alternatively, the alkaline comet assay provides a very sensitive method for detecting DNA strand breaks and repair at the level of single cell. Hence, apoptosis, micronuclei, and alkaline comet assays were employed as indicators of radiosensitivity or the adaptive response.

The present study has shown no difference in the spontaneous levels of micronuclei or apoptosis between inhabitants in high natural radiation areas and the control group. However a higher spontaneous level of DNA damage was found in the former group, indicating the presence of more alkaline labile sites, probably associated with cumulative exposure to high back ground radiation. Following the challenging dose, an inverse correlation between induced micronuclei and apoptosis was noted in the case group; lower levels of micronuclei and higher numbers of apoptotic cells. These results could be a reflection of the different levels of damage checkpoint during mitotic progression. Concerning single stranded breaks, the residents in HBRAs have shown to be more susceptible to DNA damage compared with the control group. Moreover, the rate of DNA repair was more pronounced in the case group. Data obtaining by other researchers indicate that the adapting dose can lead to a reduction of initial damage evoked by the challenging dose,[44–45] influence the progression of the cell cycle[46–47] and the incensement of apoptosis.[48–50] Additionally, the cellular manifestations of radio adaptation have been verified by the decrease in the frequency of cells with micronuclei, increase in apoptotic cell death and overall increase in the cellular repair of radiation induced DNA strand breaks.[21–22, 24,51–53]. The close relation between radiosensitivity and the overall increase in DNA damage in HBRAs has been reported previously,[54–55] and also in this study. The enhanced DNA repair and higher apoptosis in this group could be related to the induction of the radio-adaptive response in these individuals. As apoptotic cells could initiate during the early hours after irradiation,[56] the observed higher levels in the rate of DNA repair could also be due to the loss of damaged cells by apoptosis. The results are in line with the low dose hypersensitivity and induced radioresistance observed by other researchers such as Joiner *et al*.,[53] Cregan *et al*.[48] and Lambin.[49] Radioadaptive response is a biological defense mechanism in which low-dose ionizing irradiation elicits cellular resistance to the genotoxic effects of subsequent irradiation. However, its molecular mechanism remains largely unknown. A possible molecular mechanism of the adaptive response to low-dose ionizing irradiation has been discussed in relation to the repair of DNA strand breaks.[52]

Briefly, the occurrence of lower levels of micronuclei, higher numbers of apoptotic cells and enhanced DNA repair in the case group, following exposure to the challenging dose, could be related to the induction of an adaptive response in this group. However higher spontaneous levels of DNA damage found in the residents of HBRAs should indicate the presence of more alkaline labile sites, most likely associated with cumulative exposure to high back ground radiation. In addition, residents in HBRAs have shown to be more susceptible to DNA damage and thus possess more pronounced DNA repair system.

ACKNOWLEDGEMENTS

We would like to thank all the volunteers especially the inhabitants of Talesh Mahaleh, Chaparsar, and the Abe-siah quarter of Ramsar for their helpful co-operation. We would also like to acknowledge the assistance of staff at the Health Center of Ramsar in the blood sampling procedures. The authors also wish to thank Dr. Parvin Shariati for English editing.

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Received on October 2, 2005
1st Revision received on March 27, 2006
2nd Revision received on July 19, 2006
Accepted on July 19, 2006
J-STAGE Advance Publication Date: September 21, 2006