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ORIGINAL PAPER

The Cosmic Silence experiment: on the putative adaptive role of environmental ionizing radiation

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Abstract Previously we reported that yeast and Chinese hamster V79 cells cultured under reduced levels of background environmental ionizing radiation show enhanced susceptibility to damage caused by acute doses of genotoxic agents. Reduction of environmental radiation dose rate was achieved by setting up an underground laboratory at Laboratori Nazionali del Gran Sasso, central Italy. We now report on the extension of our studies to a human cell line. Human lymphoblastoid TK6 cells were maintained under identical in vitro culture conditions for six continuous months, at different environmental ionizing radiation levels. Compared to "reference" environmental radiation conditions, we found that cells cultured in the underground laboratories were more sensitive to acute exposures to radiation, as measured both at the level of DNA damage and oxidative metabolism. Our results are compatible with the hypothesis that ultra-low dose rate ionizing radiation, i.e. environmental radiation, may act as a conditioning agent in the radiation-induced adaptive response.

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Introduction

All humans receive some radiation exposure, both from natural sources, such as cosmic rays and radioactive decay products of radon gas, and from man-made sources, as in diagnostic radiology. The estimation of risk for radiation-induced cancer at low doses is commonly based on the assumption that there is a linear no-threshold (LNT) relationship between dose and risk. According to this model, risk is extrapolated linearly from epidemiological data at intermediate-/high-radiation doses (ICRP 2007) to very low doses and adjusted for dose and dose-rate effects. However, phenomena like adaptive response, genomic instability, and bystander effects may imply a deviation from this model (Huang et al. 2007).

Adaptive response refers to the ability of cells that were pre-exposed to low doses of radiation, or chemical mutagenic agents, to acquire resistance to moderate or high doses of the same or a different agent. Adaptive responses were observed in several systems in response to a number of different cytotoxic agents (Wolff 1992). The first experiments on the induction of adaptation by low, chronic doses of ionizing radiation were carried out on human lymphocytes (Olivieri et al. 1984).

However, few data exist about adaptive effects after exposure to low doses and very low dose-rates (Elmore et al. 2008). Environmental background radiation represents a source of chronic low dose rate exposure to a genotoxic agent and may be acting as a ubiquitous adaptive agent. To clarify this aspect it is essential to evaluate the risk of chronic occupational radiation exposure, as well as to understand the role of environmental background radiation in the evolution of life on Earth. The Gran Sasso National Laboratory (LNGS) of the Italian Institute for Nuclear Physics (INFN) located under the Gran Sasso mountain range in central Italy offers a unique opportunity to investigate whether a significant reduction in the background radiation exposure level can influence the susceptibility of cells to damage induced by acute exposures to genotoxic agents. The laboratory was excavated along the highway tunnel crossing the Gran Sasso Massif and is covered by at least 1,400 m of overlying rock, for an excellent shielding against cosmic rays and neutrons, fluence being reduced by a factor of 10^6 and of 10^3, respectively (Belli et al. 1989; Rindi et al. 1998).

Two previous radiobiological studies on adaptive response were performed in the Gran Sasso Laboratory: a first experiment on the yeast strain *S. cerevisiae* indicated that cells grown inside the Gran Sasso Laboratory were less efficient in repairing acute damage induced by genotoxic agents than those cultured in "reference" background radiation environment (Satta et al. 1995); in the second experiment, we used Chinese hamster cells of the V79 strain, and we reported that cells grown in low background radiation environment showed an increased apoptotic activity after treatment with cycloheximide and a greater sensitivity to mutation induction by γ-rays exposure (Satta et al. 2002).

These previous works support the hypothesis of an adaptive response manifested in the cells cultured in the external laboratory and caused by "reference" background radiation; such an adaptive response would be lost in cells cultured in a significantly reduced background radiation environment.

It is well known that ionizing radiation induces the production of reactive oxygen species (ROS) in a variety of cells (Spitz et al. 2004), and that such species may be key mediators of most biological effects of ionizing radiation. ROS are generated very rapidly through radiolysis of water molecules, and they can persist within cells participating in delayed effects (Tulard et al. 2003). However, cells are endowed with antioxidant enzymes to prevent the accumulation of ROS. These enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD reduces superoxide anion to hydrogen peroxide and molecular oxygen. The removal of hydrogen peroxide is catalyzed by the action of catalase and GPx. GPx also reduces organic hydroperoxides (Scandalios 2005).

The potential of antioxidants to reduce the cellular damage induced by ionizing radiation has been studied in animal models for more than 50 years. The application of antioxidant radioprotectors to various human exposure situations has not been extensive due to their toxicity, although it is generally accepted that endogenous antioxidants, such as cellular non-protein thiols and antioxidant enzymes, provide some degree of protection (Weiss and Landauer 2003).

The radiosensitivity of eukaryotic cells at low doses is influenced by DNA damage and repair. In vitro work using cultures of human fibroblasts indicated that damage caused by exposure to low doses of ionizing radiation may be left unrepaired (Rothkamm and Loebrich 2003). However, the result was not replicated in the clinical setting, when lymphocytes derived from humans subjected to CT scans indicated that DNA damage, measured in the form of γ-H2AX foci, was repaired completely (Loebrich et al. 2005). Whether chronic ultra-low dose-rate exposure impedes or even stimulates the DNA repair machinery is not clear. Low dose-rate exposure to γ-rays was shown to reduce DNA damage, as measured via the micronucleus assay, to a level below that of spontaneous background in primary human fibroblasts (de Toledo et al. 2006). A problem with ultra-low dose effects is that a direct measurement of DNA damage yields at low doses is difficult. However, the response of a cellular system to higher doses may act as readout to detect fine biochemical differences which may be directly influenced by low doses.

The aim of the present study was to give further support to our hypothesis of an adaptive response induced by natural background radiation. For this purpose, we investigated the biological response of human lymphoblastoid cells TK6 to acute exposures to X-rays. Cultures were maintained in parallel, for 6 months, at the Istituto Superiore di Sanità (ISS), in a "reference" background radiation environment, defined as "external laboratory", and at the LNGS, in a low background radiation environment, defined as "underground laboratory". The biological end-points considered were growth rate, DNA damage, and antioxidant enzymatic activities.

Materials and methods

Dosimetry and irradiations

Background radiation dosimetry was evaluated from three major sources: α-particles from ^{222}Rn and its daughters, cosmic rays, and terrestrial γ-rays. To estimate the ^{222}Rn dose component, measurements of ^{222}Rn concentration in air were obtained using a Radon meter and converted to radiation doses to cultured cells using an in vitro model (Jostes et al. 1991). Briefly, the model devised by Jostes et al. for Chinese Hamster Ovary cells exposed to moderately high activities of ^{222}Rn was applied here to the case of

TK6 cells at environmental ^{222}Rn activity levels. To convert from environmental activity to dose to the cells, secular equilibrium was assumed with daughter products of ^{222}Rn decay (^{222}Rn: ^{218}Po: ^{214}Po). Further, we assumed that ^{222}Rn concentrations in air and liquid phases, dissolved in the culture medium, were at equilibrium and that the ratio of ^{222}Rn activity in the liquid phase to the activity in the air phase is 0.167 at 37°C (M. G. Pugliese, personal communication). Importantly, to prevent accumulation of ^{222}Rn activity indoors, the LNGS laboratories were equipped with a powerful air ventilation system that collects air from outside the highway tunnel, on the Teramo province side, and expels indoor air outdoors. Terrestrial and cosmic γ-rays (this latter component being appreciable only for the external laboratory) were measured directly using TLD 700H detectors.

Radiosensitivity tests were carried out by X-irradiation at San Salvatore Hospital at Coppito in the L'Aquila area, at 2 Gy min^{-1}, using a 6 MV medical linear accelerator. This facility is located at a distance of about 20 km from both culturing places; total traveling time to and from the irradiation facility plus duration at the facility was always below 2 h.

Cell culture

TK6 lymphoblastoid cells were obtained from the European Collection of Cell Cultures (ECACC, cat no 95111735) and grown as suspension cultures at 37°C in 5% CO_2–95% air in RPMI 1640 medium supplemented with 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin, 2 mM L-glutamine, and 10% v/v horse serum, heat-inactivated for 2 h at 56°C (all reagents from Invitrogen). Cells were subcultured three times a week at 5×10^4 mL^{-1} or 1×10^5 mL^{-1} and reached a maximum concentration of 1.3×10^6 mL^{-1}. To minimize deviations in culture conditions, all reagents as well as tissue culture plastics were identical in both the LNGS and ISS labs. Mycoplasma tests were routinely run during the continuous culture in both laboratories. To reduce the risk of isolating a mutant clone with a growth advantage in either laboratory, two parallel, independent cultures were maintained in each laboratory and treated always separately at each passage. Therefore, all experimental determinations were conducted on a total of four cultures separately (external laboratory: denominated A and B; underground laboratory: denominated C and D). Samples of the ongoing, continuous cultures were frozen in liquid nitrogen every 30 days for experimental archival.

Growth curve

For determination of growth rate, cells were sub-cultured at a concentration of 1×10^5 mL^{-1}, sampled at regular time intervals, and their concentration measured using a Coulter counter. Determinations in the exponential growth phase were subjected to regression analysis and their doubling time estimated.

Micronucleus assay

For the measurement of DNA damage and repair at the level of micronuclei induction, the methods by Fenech and collaborators were adopted (Fenech et al. 2003). Briefly, after 2 Gy irradiation at room temperature, cells were grown at 37°C for 24 h in the presence of 3 μg mL^{-1} cytochalasin B (Sigma), before being washed with PBS and fixed in ice-cold methanol:pure acetic acid (9:1, v/v) for 20 min. Microscope slides were prepared with the fixed suspensions and stained with DAPI for visualization of micronuclei on a fluorescent microscope. At least 2,000 binucleated cells were scored for each sample. All cells bearing one or more micronuclei were considered micronucleated events (the frequency of binucleated cells bearing more than one micronucleus being very low, in the order of 2 per 1,000).

Cell extract preparation and enzymatic activity assays

Control and irradiated (1 Gy, room temperature) cells were harvested at ice-cold temperature and re-suspended at the concentration of 10^7 cells mL^{-1} in 10 mM phosphate buffer, pH 7.0, containing 10 mM dithiotreitol (DTT, for glutathione peroxidase enzyme) or Triton X-100 (for catalase and total super-oxide dismutase enzymatic assay). All chemicals were purchased from Sigma unless otherwise specified. Cell suspensions were thawed–frozen three times in liquid N_2, homogenized and centrifuged at 13,000 rpm for 30 min at 4°C. The resulting cell extracts were used for spectrophotometrical measurement of enzymatic activity and protein content.

Total superoxide dismutase (SOD, EC 1.15.1.1) activity in cell extracts was assayed at 480 nm and 30°C by its ability to inhibit the epinephrine autoxidation, according to Sun and Zigman (1978). The reaction was carried out in 50 mM sodium carbonate buffer, pH 10.2, and was initiated by the addition of 0.1 mM adrenaline. A standard curve, with a purified Cu-Zn bovine SOD, was obtained by plotting the inverse values of the amount of enzyme used and the percentage inhibition observed. This standard curve was used to determine the amount of extract necessary for a 50% inhibition. One unit of SOD was defined as the amount of the enzyme required to halve the rate of epinephrine autoxidation.

Catalase (CAT, EC 1.11.1.6) activity was measured at 240 nm and 25°C by following the rate of reduction of hydrogen peroxide, according to Aebi (1984). The reaction

mixture contained 100 mM potassium phosphate buffer pH 6.8 and 10 mM H_2O_2. One unit of CAT is defined as 1 μmol of H_2O_2 reduced per minute.

Selenium-dependent glutathione peroxidase (Se-GPX, EC 1.11.1.9) activity was assayed according to Paglia and Valentine (1967). The assay solution contained 50 mM monobasic potassium phosphate, pH 7.0, 1 mM EDTA, 1.5 mM sodium azide, 0.4 U glutathione reductase, 0.45 mM GSH, 0.2 mM NADPH and 0.25 mM H_2O_2 as substrate. The oxidation of NADPH was followed at 340 nm and 25°C. One unit of SeGPX was defined as 1 μmol of NADPH oxidized per minute.

Protein concentration was determined by Protein Assay Kit (Bio-Rad) (Bradford 1976), using bovine serum albumine (BSA) as standard.

Statistical analysis

Micronuclei frequency data were analyzed for statistical significance using the Pearson χ^2 test on 2×2 contingency tables with one degree of freedom.

Enzymatic activities data were analyzed for statistical significance using Student–Newman–Keul's test (SigmaStat software; Jandel Scientific Software Corporation, San Rafael, CA, USA).

Results

Dosimetry

The average ^{222}Rn concentration measured in the underground laboratory at the site of the cell incubator was equal to 5 Bq m^{-3} and that in the external laboratory was 50 Bq m^{-3}. Based on the assumptions and procedures made in the dosimetric model described in "Materials and methods", the calculated dose-rates to the cells were 0.17 and 1.7 nGy h^{-1}, respectively. The γ-ray dose-rates were measured to be 3.6 and 300 nGy h^{-1} at the underground and at the external laboratory, respectively. Cosmic radiation was considered negligible for the underground laboratory, and equal to 30 nGy h^{-1} at the external laboratory, based on UNSCEAR 2000 data (Sources, Annex E). Even if these are crude estimates, more precise evaluation of this component would have little impact on the overall dosimetry. Overall, background radiation dosimetry in the underground laboratory showed about 87-fold lower total level of background dose-rate compared to the external laboratory. Table 1 summarizes the dosimetry measurements and estimations for both laboratories.

It has to be considered that changes in the relative contribution of radon and cosmic rays may change the high linear energy transfer (LET) component, which is known to have higher biological effect than low LET radiation. Radon and its decay products produce high LET alpha particles, and there is a significant high-LET component in cosmic rays also at ground level. It is evident that because of shielding of the high LET cosmic component, only ^{222}Rn and its decay products contribute to high-LET dose in the underground laboratory.

Table 1 Dosimetry estimates

Background radiation component	Reduced background (underground) (nGy/h)	Normal background (external) (nGy/h)
^{222}Rn and daughters[a]	0.17	1.7
All γ-rays[b]	3.6	300
Cosmic rays	Negligible	30[c]
Total dose-rate	3.8	331.7

[a] Based on the application of the model by Jostes et al. (1991)

[b] TLD measurements

[c] Based on UNSCEAR 2000 Report, Sources, Annex E

Growth curves

At the beginning of the experiment and after 6 months of continuous, parallel culture, cell growth was characterized via a growth curve. For the cell culture that was used to start the experiment (denominated "t_0" culture), prior to dividing it into two parallel cultures at LNGS or ISS, the measured doubling time was 16.4 ± 0.1 h. After 6 months there were no apparent differences in cell cycle duration among the four cultures, the doubling times being 16.4 ± 0.1 h (culture A), 16.6 ± 0.3 h (culture B), 16.7 ± 0.1 h (culture C), and 16.3 ± 0.1 h (culture D).

Micronucleus assay—DNA damage

Figure 1 shows the results of the micronucleus assay, conducted after 6 months of continuous culture at either laboratory, based on two independent experimental repeats. Also shown is the micronuclei induction in the t_0 culture. For all these conditions, micronuclei frequencies measured in the absence of a challenge dose are also shown (baseline levels).

Results showed that, after 6 months of parallel cultures, both cultures in the underground laboratory (C and D) present higher micronuclei yields after a challenge dose of 2 Gy, compared to both cultures held at reference background radiation dose rate (A and B). When comparing either baseline or X-irradiated levels, respectively, Pearson's χ^2 tests indicated that micronuclei frequencies for cultures A and B were not statistically different from each other. The same applies to cultures C and D. However, and for X-irradiated cultures only, either A or B micronuclei frequencies are statistically different from frequencies measured

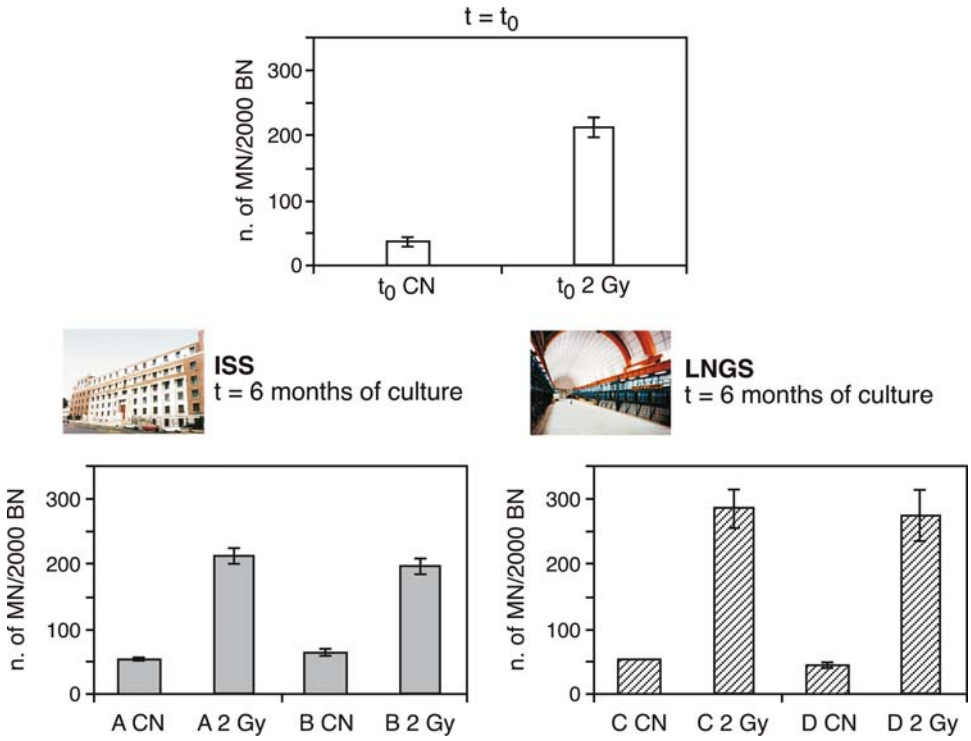

Fig 1 Number of micronuclei in 2000 binucleated cells induced by 2 Gy X-irradiation; comparison with the corresponding control is also shown. t_0: *white bars*, ISS cultures: *gray bars*, LNGS cultures: *dashed bars*. *Bars* represent the mean of two experiments with SE

for either culture C or D ($P < 0.05$), suggesting that reduced environmental background radiation acts as if removing a natural, chronic adaptive effect.

Antioxidant enzymatic activities

The enzymatic activities of total superoxide dismutase (SOD), catalase (CAT), and selenium dependent glutathione peroxidase (Se-GPx) were assayed in TK6 cells at zero time and after 6 months of continuous culture in both laboratories. In preliminary experiments, the antioxidant enzymatic activity levels were measured after 1, 3 and 6 h of incubation at 37°C post-irradiation with an X-ray dose of 1 Gy. Since variations in enzymatic activities were observed only at 1 h, all further experiments were performed 1 h after irradiation.

As shown in Fig. 2, SOD enzymatic activity was lower after 6 months of culture ($P < 0.001$) compared to t_0 cells for both environmental culture conditions. Moreover, a significant decrease ($P < 0.001$) in CAT and Se-GPx enzymatic activity was found after 6 months in both cultures in the underground laboratory (C and D), not only with respect to the external cultures (A and B) but also with respect to the t_0 culture.

Comparing cells grown for 6 months in both laboratories exposed to 1 Gy "challenge" dose with their respective controls, exposure to X rays did not affect SOD and CAT enzymatic activities. By contrast, after irradiation, the specific activity of Se-GPx showed an increase ($P < 0.01$) in both cultures maintained at the external laboratory for 6 months, but not in the underground cultures. A significant decrease ($P < 0.01$) was found after irradiation in the t_0 culture.

Cell sensitivity to a ROS attack depends on the relationship between CAT or GPx and SOD, rather than on absolute amounts of individual antioxidant enzymes. Therefore, the ratios CAT/SOD and Se-GPx/SOD are indicators of the scavenging efficiency of cells against ROS. A decrease in one or in both of these ratios indicates a poor ROS scavenging efficiency (Somani et al. 1996). In unirradiated controls, a significant reduction ($P < 0.001$) of both ratios was observed in the cultures held in the underground laboratory either with respect to the external cultures or with respect to the t_0 culture (Fig. 3). Moreover, after X-irradiation, cells grown for 6 months in the external laboratory showed a significant increase of Se-GPx/SOD ratio ($P < 0.001$) while no differences were observed in cells grown in the underground laboratory compared with their respective control. It is interesting to note that irradiated t_0 cells reveal a strong reduction of both ratios compared to their non-irradiated control.

These results suggest that cells maintained in the presence of "reference" background radiation are more efficient in removing ROS than those cultured in a low background radiation environment.

Discussion

Using a yeast model (Satta et al. 1995) and a rodent cell line model (Satta et al. 2002), we previously obtained

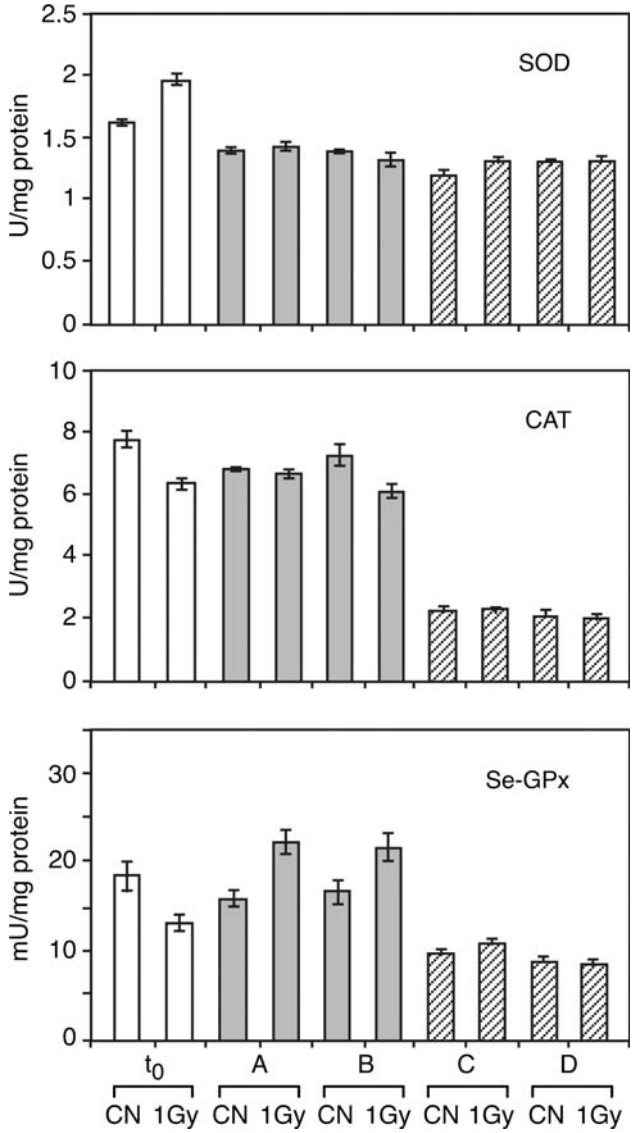

Fig 2 Antioxidant enzymatic specific activities in control (CN) and 1 h after irradiation (1 Gy X-rays) TK6 cells: at zero time (*white bars*, t_0), after 6 months of growth at reference background radiation (ISS: *gray bars*, A and B cultures), and at low background radiation (LNGS: *dashed bars*, C and D cultures). Values are mean ± SEM of three different experiments

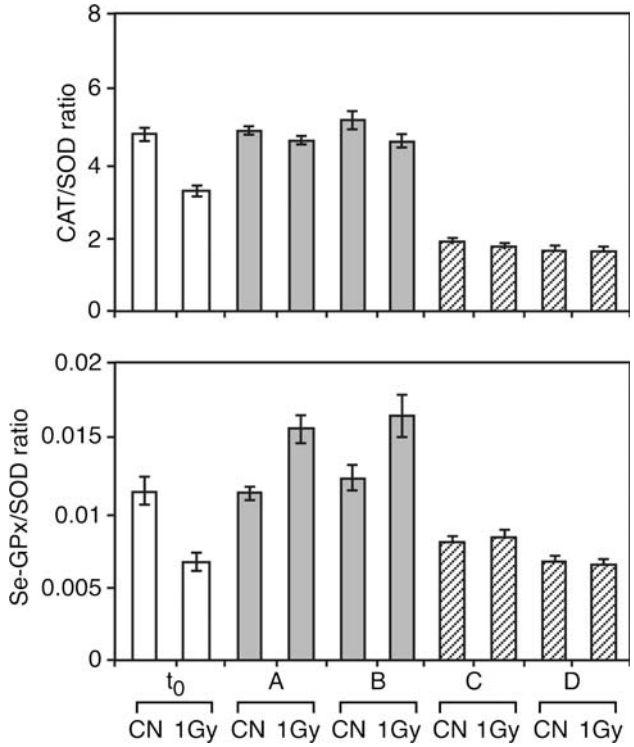

Fig 3 CAT/SOD and Se-GPx/SOD ratios in control (CN) and 1 h after irradiation (1 Gy X-rays) TK6 cells: at zero time (*white bars*, t_0), after 6 months of growth at reference background radiation, (ISS: *gray bars*, A and B cultures), and at low background radiation, (LNGS: *dashed bars*, C and D cultures). Values are mean ± SEM of three different experiments

indications that reduced environmental background radiation levels render cells less competent in coping with insults conferred by genotoxic agents, including ionizing radiation. These results are compatible with the idea that low dose rate environmental background radiation may be acting as a *conditioning* agent. In this new work we have extended our previous observations to a human lymphoblastoid in vitro cell culture model.

Our results indicate that cell doubling times did not vary for all cell cultures, as assayed at 6 months of protracted culture, from the beginning of the experiment. This evidence is in agreement with our previous determinations in V79 cells, where no alterations were detected in the doubling times of the different cultures (Satta et al. 2002).

Micronuclei are a well-known indicator of unrepaired or mis-repaired chromatin damage. In our study, maintenance under different levels of environmental background radiation did not alter the spontaneous rate of micronuclei formation. However, upon challenging both TK6 cultures with 2 Gy X-radiation, we observed higher yields of micronuclei in the cells that had before been maintained under reduced levels of environmental ionizing radiation, compared to cells cultured under reference conditions. This is suggestive of an adaptive response induced by chronic low dose rate exposure from environmental background radiation.

Exposure of cells to ionizing radiation leads to the formation of ROS that are associated with radiation-induced cytotoxicity. ROS show high reactivity to a variety of cellular macromolecules including DNA, proteins, and lipids (Spitz et al. 2004; Scandalios 2005). Cells possess several mechanisms for the maintenance of redox balance (redox homeostasis), even after temporary exposure to increased concentration of ROS, which is maintained by antioxidant defense systems. The imbalance of the oxidative state triggers redox-sensitive signaling pathways, which in the case of excess of ROS may lead to the induction of antioxidant

activities. It is well demonstrated that antioxidant defenses may play a crucial role in protecting cells against ROS injury during ionizing radiation exposure (Sun et al. 1998), and that the efficiency of signal transduction pathways activated by oxidative stress declines with aging (Miura 2004). Since aging in all our TK6 cell cultures is the same, we should observe the same decline in ROS-scavenging efficiency in all cultures. However, in our experiments we found that cells grown in reference background radiation conditions appear sufficiently protected against elevated ROS production due to the aging process, compared to those grown in low background radiation environment. The only difference that can be reasonably identified between these culture conditions is the different background radiation level. Therefore, it is likely that the presence of the reference background radiation environment induces an adaptive response in TK6 cells. In our case, factors involved in what appeared as a radiation-induced adaptive response were antioxidant defense systems that, probably, are less active when cells are grown in low background radiation environment.

In order to give a firmer support for the hypothesis of background radiation-induced adaptive response, we assayed the antioxidant enzymatic activity after an acute exposure to X-rays in TK6 cells grown for 6 months in different background radiation conditions. Our results indicate that irradiated cells grown in the external laboratory exhibit an increase of ROS-scavenging efficiency as measured by CAT/SOD and GPx/SOD ratios with respect to their control. By contrast, irradiated cells grown in the LNGS underground laboratory are not protected against ROS produced by exposure to ionizing radiation. It is possible that growth in a reference background radiation environment, as at ISS laboratory, causes an imbalance in redox homeostasis and triggers redox-sensitive signaling pathways, resulting in the induction of antioxidant defense systems and the acquirement of resistance to subsequent high-dose challenge irradiation. Although the amount of damage induced by X-irradiation may be the same in all cell cultures, the difference in scavenging efficiency found between the reference and the "reduced" background radiation could be ascribed to the induction of an adaptive response induced by the reference background radiation.

In conclusion, our results provide evidence that the response of TK6 cells, cultured for 6 months in a reference background radiation environment, is different from that in the same cells cultured under similar condition in a low radiation background environment. These results indicate a more rapid scavenging of ROS and, perhaps consequently, less DNA damage when assayed at the level of micronuclei. This phenomenon belongs to those currently defined as adaptive response.

Other investigators have attempted to evaluate the adaptive effects of chronic exposure to low dose-rate ionizing radiation. A recent work by Elmore et al. (2008) suggests that low dose-rate irradiation at 1.4 mGy per day (three orders of magnitude above the dose rate in our study), protracted for 3 months up to a total dose of 216 mGy, can confer an adaptive response against acute exposures to γ-rays in a hybrid HeLa/human fibroblast in vitro cell model, using neoplastic transformation as endpoint. These authors also showed that in the cells conditioned at this dose rate (but not challenged by a subsequent acute dose), neoplastic transformation was reduced below the spontaneous level, but when the dose rate was reduced below 1 mGy per day this effect was lost. This result was interpreted as suggestive of a loss of the adaptive response at a dose rate below 1 mGy per day. Therefore, it would be interesting to assess whether chronic exposures above background radiation levels, and below 1 mGy per day, can elicit an adaptive effect after a challenging dose.

The indications given by the present study about possible radioadaptation due to the natural radiation background confirm the need for elucidating the mechanisms underlying adaptation when chronic ultra low dose rate exposures are involved, and suggest future research directions for improving our understanding of the role of adaptive responses in view of possible impact to human risk assessment.

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