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Effects of deprivation of background environmental radiation on cultured human cells

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Summary. — In this paper we present results from an experiment aimed at investigating whether living cells are influenced by background ionizing radiation. Parallel human cell cultures were set-up in two separate laboratories and maintained for several months under identical conditions but for a 80× different level of background ionizing radiation. Periodically, the cell cultures were monitored for the onset of divergences in biochemical behavior, using two distinct cellular biology assays, namely micronuclei induction and activity of enzymes implicated in the management of oxidative stress. To reveal any subtle modifications, responses were also amplified by subjecting cell cultures to acute stress induced by exposure to moderately high doses of ionizing radiation. Compared to reference radiation background conditions, cultures maintained in a reduced background radiation environment handled the consequences of acute stress with diminished efficacy.

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1. – Introduction

Life has evolved on Earth for 3 billion years in the presence of background ionizing radiation. Living organisms may keep memory of being in the presence of ultra low dose rate radiation, so that a question arises as to whether the biochemical behavior of living organisms and life on Earth would differ in the absence of radiation [1, 2].

To address this scientific question, an ideal experimental design would consist in the twin set-up of a cell or animal culture in a laboratory where background radiation is reduced as low as possible, and in a reference laboratory at "normal" background levels. Using a number of experimental assays, the experimenter would monitor the biological model for the onset of any differential behavior between the two laboratories. To achieve a condition of reduced environmental radiation, a suitable location is the underground laboratory of the Italian National Institute for Nuclear Physics at Gran Sasso (LNGS/INFN), located in central Italy alongside a highway tunnel between the cities of L'Aquila and Teramo, underneath at least 1400 m of limestone rock. This unique setting allows a significant reduction in the muon flux [3] and the virtual elimination of atmospheric showers [4]. Inside the underground laboratory, the organic nature of limestone rock results in a greatly reduced environmental background radiation [5]; moreover, the laboratory walls are lined with specifically chosen low-activity concrete. To minimize accumulation of ^{222}Rn which would result in a substantial exposure to densely ionizing, and therefore highly biologically effective, radiation, the underground laboratory is equipped with a ventilation system that captures air from outside the highway tunnel and pumps it inside the laboratories. Occasional and short-term controlled shut-down of such ventilation system can result in spikes of ^{222}Rn activity.

In this work, the "reference" laboratory was established at Istituto Superiore di Sanità near central Rome, such that the reduction of environmental radiation level achieved, relative to the reference laboratory, is 70-fold, but reaches 80-fold when a low-activity iron shielding is adopted inside the underground laboratory [6].

Unlike most physics experiments, our biophysics experiment is focused on measurements on biological, living systems. The terrific complexity of a living being, even at the cellular level, affects the accuracy of any experimental determination. To minimize the effects of the large number of variables involved, one needs to define a suitable experimental model which offers control over the variable of major interest, while attempting to diminish the role of potential confounders. For preliminary investigations in which one needs to isolate and focus on few variables, *in vitro* cell culture systems are typically at an advantage over more complex animal models, and we have accordingly designed and conducted a series of experiments in several *in vitro* models [7, 8]. Our previous experiments suggested that cells grown in reduced background radiation conditions manifested an altered response to acute doses of ionizing radiation or exposures to genotoxic agents such as methyl-methan-sulphonate (MMS) [7, 8]. Specifically, cells grown in reduced background radiation conditions were less capable of facing the damage induced by such agents, relative to cultures maintained in reference background conditions (outdoors). Markers of such deviations included gene mutations, DNA damage as measured by micronuclei induction [9], activity of enzymes involved in the management of oxidative stress [10-12], and cell cycle duration.

Stemming from our past experience, we have recently set up an *in vitro* experiment whereby parallel cultures of human lymphoblastoid TK6 cells were maintained in identical experimental conditions with the exception of background radiation. As our earlier measurements revealed different responses between underground and above-ground

cultures of *S. Cerevisiae* and Chinese hamster V79 cells only after several cell generations [7,8], we set out to carry on the human lymphoblastoid cultures for up to continuous 12 months in both laboratories [6].

At regular time intervals, the on-going cell cultures were subjected to multiple measurements, to detect any signs of biochemical deviations due to different levels of environmental radiation, in terms of duplication time, ability to cope with endogenous DNA damage via a "micronucleus" assay, and ability to maintain redox homeostasis via an enzymatic activity assay. Additionally, at regular time intervals, cells were frozen in liquid nitrogen (LN_2) for archival of biological specimens.

Since subtle biological effects were expected after such a minimal variation of living environment, which could remain undetected by our measuring apparatus, we also set to implement a potentially more sensitive detecting test by challenging the twin cell cultures with an acute exposure to a cytotoxic agent. In so doing, it is expected that any small biological differences could be amplified and detected. Conveniently, this cytotoxic agent was ionizing radiation itself, imparted at doses about three orders of magnitude larger than exposures caused by environmental radiation.

While the experiment is still in progress, we here report on preliminary measurements performed after six months of continuous cell culture in both laboratories, hinting at significant effects of deprivation of background radiation. We observed lower repair efficiency of DNA damage caused by acute exposure of radiation in the underground, environmental radiation-deprived cultures, and altered management of redox environment as a function of background radiation levels.

2. – Material and methods

Experimental methods were described in detail elsewhere [6]. Briefly, human lymphoblastoid TK6 cultures were maintained in standard conditions at 37 °C in a cell culture CO_2 incubator for up to six months. All plastic and reagents were kept identical at both cell culture sites to minimize any laboratory effect on the responses sought. To control for any genetic drift that could result, during the six months of continuous culture, in the isolation of genetic mutants that may bias the experimental observations, we set up two independent cell cultures at each laboratory site, and conducted all experiments on each "sister" cell culture separately and concomitantly. With this approach, it is highly unlikely that genetic drift could have acted in the same fashion in both sister cultures.

For challenge experiments, exposures to acute doses of ionizing radiation were carried out at an external facility in the city of L'Aquila. In these instances, exposure to normal environmental background radiation, including cosmic radiation, was negligible.

All cultures were handled similarly and experimental assays were run on coded samples, with the code unknown to the person that conducted the terminal measurements.

3. – Assessment of chromosomal damage

Some DNA damage events can lead to morphological changes in the nuclear DNA that may be readily visualized using conventional fluorescence microscopy techniques. When a damaged DNA fragment, detached from a chromosome, is produced, it may be lost from the nucleus when a cell attempts to undergo mitotic division. The micronucleus assay [9] allows the visualization of these events by blocking cell division before such fragments go lost. At the microscope, the detached DNA fragment will appear as a fluorescent

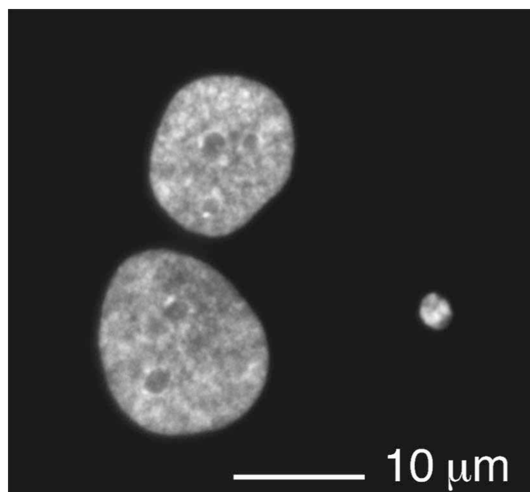

Fig. 1. – A typical image captured at the fluorescence microscope, showing two nuclei of a duplicating cell with a "micronucleus".

spot, similar to a cell nucleus, but about 1/10 of its size, positioned nearby two regular nuclei which the cell was attempting to segregate into two daughter cells (fig. 1). The frequency of micronucleated events, *i.e.* of binucleated cells that contain at least one micronucleus, will serve as a quantitative indicator of failed repair of damage to DNA caused by a genotoxic agent, in our case ionizing radiation. The experimental protocol for preparation and scoring of micronuclei events was described elsewhere [6, 9].

4. – Measurements of activity of enzymes involved in the management of reactive oxygen species

Regular cell functions require maintenance of several balance states. One of such equilibrium states is that of chemical species that are involved in reduction and oxidation of other species. Deviations from a state of equilibrium may generate a range of cellular effects. In particular, accumulation of reactive oxygen species (ROS) can be readily generated by acute exposures to ionizing radiation and the efficacy of their clearance is an indicator of a cell's health. An established method to evaluate the efficacy of ROS clearance is the direct measurement of the activity of enzymes implicated in ROS management. In particular, we have measured the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) using methods that have been described elsewhere [6]. Since the aforementioned enzymes act in concert, measurements of enzymatic activity are also accompanied by activity ratios that provide additional indication of ROS scavenging efficacy.

5. – Results

5˙1. *Chromosomal damage.* – The frequency of micronuclei was determined for all the cultures maintained for six months under reduced and reference background radiation (two independent determinations, hereon denominated "runs"), as well as for the cell culture that was used to generate the previous two when the experiment was first

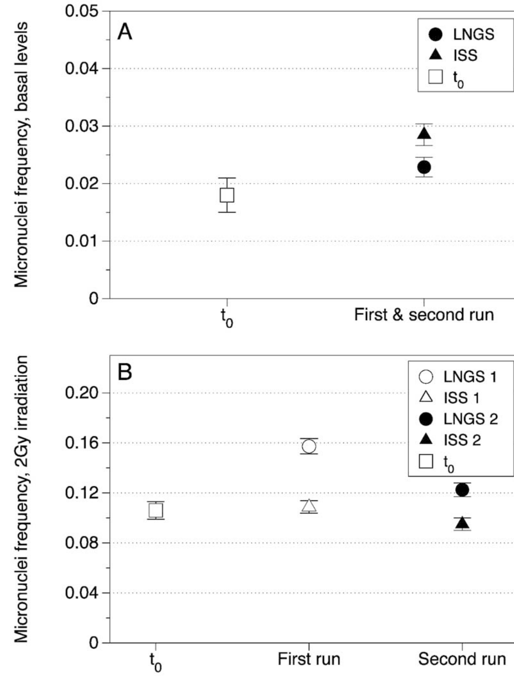

Fig. 2. – Frequency of micronuclei events in basal (A) and 2 Gy-irradiated cell cultures (B) maintained for six months under reduced (LNGS) and reference (ISS) background radiation, as well as for the cell culture that was used to generate the previous two when the experiment was first set up (t_0). Error bars represent standard errors, assuming Poisson's statistics. Experimental runs have been indicated with numbers 1 and 2 in the legend of panel B.

set-up (denominated "t_0"). Contingency tables were built, and subjected to a χ^2 test, to verify whether measurements from independent samples were not statistically different, in which case they were cumulated to increase precision. This was the case for the basal frequency of micronuclei events (an estimate of lack of repair or mis-repair of spontaneous chromosomal damage) for both cultures maintained in reduced and standard background environment. Measurements indicate an increase with time in both laboratories compared to the initial level (t_0 value; panel A, fig. 2). However, this increase is statistically significant only for the cell culture maintained for 6 months in reference radiation background conditions (ISS, $p = 0.009$). These data show that cells maintained under reduced background conditions for 6 months display a limited increase of spontaneous level of DNA damage, relative to cells maintained in reference conditions.

Maintenance of cell cultures in reduced background environment may cause subtle effects that could remain undetected by this technique. To amplify any such effects, we used the same technique to determine the micronuclei frequency in cells that were exposed to an acute dose of 2 Gy of ionizing radiation, *i.e.* large enough to produce extensive DNA damage [13]. The results are reported in fig. 2 (panel B). For acutely irradiated cultures, micronuclei frequency determinations were not cumulated from independent runs ($p > 0.05$). Results indicate that maintenance of cells under reduced background radiation environment increases the frequency of micronuclei that is caused by the acute radiation exposure, relative to our reference background radiation conditions (first run 1.45 ± 0.09

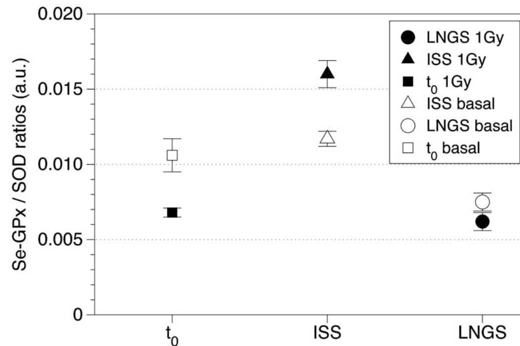

Fig. 3. – Ratios of Se-GPx to SOD measured activity, expressed in arbitrary units, for basal and 1 Gy-irradiated cell cultures maintained for six months under reduced (LNGS) and reference (ISS) background radiation, as well as for the cell culture that was used to generate the previous two when the experiment was first set-up (t_0). Error bars represent standard errors.

fold, $p < 0.05$; second run 1.29$\pm$0.09 fold, $p < 0.05$). Moreover, the micronuclei frequency measured in cells maintained under reference background conditions is consistent with that measured at t_0 (first run 1.03 ± 0.09 fold, $p > 0.05$; second run 0.90 ± 0.08 fold, $p > 0.05$). Given that the amount of DNA damage caused must be identical (the damage caused by the low level exposure to background ionizing radiation being negligible), this enhancement in micronuclei frequency reflects a reduced efficacy of DNA repair activity. Such effect may have been caused by the reduction of background radiation. Taken together and albeit the variability across the two experimental runs, this experimental evidence shows that, at the level of repair of DNA damage, the behavior of the cells maintained under reduced radiation background for prolonged times is different from that of reference cultures.

5·2. *Management of reactive oxygen species*. – Measurements of SOD, CAT, and GPx activities were carried out on both cell cultures, maintained for six months under the two different background radiation environments and on the t_0 culture, before and after irradiation with a dose of 1 Gy. The GPx/SOD and CAT/SOD ratios may be used as estimators of the protection level against imbalance of ROS. A large value of any of these two ratios will indicate that the cell cultures are able to react against the potentially detrimental effects of excess ROS. By contrast, low values of these ratios indicate reduced ability to face ROS imbalances.

In fig. 3 we present only the measured GPx/SOD ratios for all cultures used in this experiment. Relative to the t_0 cultures, cells maintained for prolonged times in culture show an altered capacity to control cellular ROS equilibrium. Specifically, the response of t_0 cultures to acute exposure to ionizing radiation results in a reduced ROS scavenging activity, relative to constitutive/basal levels, while maintenance of cultures for six months in reference background conditions (ISS) results in the opposite effect. By contrast, such response to ionizing radiation was significantly lost in cultures chronically deprived of background radiation (LNGS).

6. – Discussion

As part of an on-going experiment, we have here reported the results of several measurements conducted on human TK6 cell cultures maintained for six months under

reduced or reference background radiation environments. We hypothesized that attenuation of background radiation levels may cause subtle biochemical changes in cell cultures, and we have therefore elected to conduct a highly controlled experiment in which all materials and methods were rigorously identical with the exception of background radiation levels. Given that six months of continuous culture results in hundreds of mitotic cell divisions, genetic drifts may have occurred in these cultures that could have rendered them differently able to respond to the experimental stimuli that we chose to employ. To control for any such drift, we set up two independent cell cultures at each laboratory site, and conducted all experiments on each "sister" cell culture separately and concomitantly. With this approach it is highly unlikely that genetic drift could have acted in the same fashion to both sister cultures. In fact, our measurements indicated that responses of sister cultures were the same, within statistical errors (not shown), so that we here presented cumulated results within each run (figs. 2 and 3).

Compared to reference background conditions (ISS), cultures maintained in a reduced background radiation environment (LNGS) handled DNA damage caused by acute exposure to ionizing radiation less efficiently (fig. 2) and were unable to react to the imbalance of ROS that follows acute exposure to 1 Gy (fig. 3).

At the level of DNA damage, basal levels of micronuclei frequencies were significantly larger in ISS cultures compared to t_0 cells (two standard deviations), while basal levels were unchanged, within errors, in LNGS cultures (fig. 2, panel A). Such difference may be caused by either an increase in the spontaneous amount of DNA damage in ISS cultures, via an augmented oxidative stress, or by an increased efficiency of the DNA repair machinery in the LNGS cultures. This second hypothesis is less plausible, since it hinges on the idea that a reduced level of damage could stimulate repair.

Acute exposures to ionizing radiation showed larger levels of micronuclei frequency in LNGS cultures than in ISS cultures, whose micronuclei frequency are the same, within errors, as those measured in t_0 cultures (fig. 2, panel B). As indicated above, constitutive oxidative stress caused by background ionizing radiation could be responsible for these observations: at such extremely low levels, oxidative stress originating from background radiation may be exerting a stimulating action on the DNA repair machinery, which, upon virtual elimination of the stimulus, as in the LNGS cultures, may be not prepared to face the consequence of exposure to a much larger dose of radiation.

At the level of ROS management in response to acute exposure to ionizing radiation (fig. 3), our measurements indicated that the cellular scavenging efficiency (as measured via the Se-GPx/SOD ratio) is diminished in t_0 cells, while it is elevated in cultures maintained for six months in reference radiation background conditions. These measurements are the result of five independent repeats and show differences larger than two standard deviations; it is unlikely that they were due to chance. However, the emerging results are puzzling and difficult to interpret. We hypothesize that the cultures maintained for six months in reference radiation background conditions have elevated ROS levels, compared to t_0 cultures. Although a different behavior is not manifest at the level of constitutive ROS scavenging activity (fig. 3), a constitutively high level of ROS may render ISS cultures more prone to react to the sudden elevation of ROS levels that results from the acute exposure to ionizing radiation. Such hypothesis of elevated constitutive ROS levels in the ISS cultures is attractive, as it could also explain the results of the measurements made with the micronucleus assay (fig. 2, panel A) as discussed earlier. Interestingly, other investigators have demonstrated that alterations of ROS levels may have significant impact on basic cellular functions [14].

By contrast, the metabolic behavior of cells maintained for six months in reduced background radiation environment is different both from the t_0 cultures and from the ISS cultures, as warranted by relative small experimental errors on these measurements. As a matter of fact, the response of LNGS cultures to acute radiation at the level of ROS management, within experimental errors, was negligible. These differences prompt us to conclude that the ISS and LNGS cultures have acquired a markedly diverging behavior (more than three standard deviations).

Taken together, experimental measurements at the level of DNA damage and repair, and management of ROS balance, strongly suggest that TK6 cell cultures develop different behaviors under reduced or reference background radiation environments. Therefore, it is likely that the presence of a normal background radiation environment favors the maintenance of protective responses in human TK6 cells. It is tempting to speculate that evolution of life on Earth may have been different in the absence of environmental, background ionizing radiation [1].

High throughput comparative analyses of the cellular transcriptome (*i.e.* the set of all RNA molecules) of cells chronically maintained under reduced background radiation, and reference conditions, are in progress using DNA microarrays and will be subject of a future report.

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