CHAPTER 32

MOLECULAR AND CELLULAR EFFECTS OF CHRONIC LOW DOSE-RATE IONIZING RADIATION EXPOSURE IN MICE

ANDREYAN N. OSIPOV

N.I. Vavilov Institute of General Genetics RAS, Gubkin str. 3, Moscow 119991, Russia
e-mail: andreyan.osipov@rambler.ru

Abstract: The results of the three independent experiments on study of molecular and cellular effects in CBA/lac male mice chronically (up to 1 year) exposed to γ-radiation at a dose rate of 61 cGy/year are reviewed in this chapter. It was shown that the DNA–protein cross-links level was statistically increased only at early terms of irradiation (up to 40 days (~6.8 cGy)). The results of the comet assay study on spleen cells showed that very low dose-rate irradiation resulted in statistically significant increase in DNA strand breaks level, starting from a dose of ~20 cGy (120 days). Further prolongation of exposure time and, hence, increase of a total dose did not, however, lead to further increase in the extent of DNA strand breaks level. A dose–response curve for DNA single-strand breaks is good fitted by a polynomial regression $y = 0.6209 + 0.0313^{*}x - 0.0004^{*}x^2$, where y is the average comet index and x is a dose in cGy. At the days 120, 270, and 365 of the chronic irradiation (20, 45, and 61 cGy, respectively), approximately twofold increase over a control level in the apoptotic cell fraction was observed. It was found that chronic action of low dose-rate γ-radiation led to a change in the sensitivity of spleen cells to H_2O_2 exposure. A weakening of cellular antioxidant potential and/or repair capacity has been observed at early terms of irradiation (up to 80–120 days). In contrast, prolongation of irradiation resulted in activation of defense system in spleen cells. This effect could be attributed to a development of adaptation processes triggered upon accumulation of a certain dose. A «bystander effect», response of unirradiated directly cells due to signaling originating from irradiated cells, can be possibly involved in the effects observed in this study.

Keywords: DNA–protein cross-links; single-strand DNA breaks; apoptosis; micronuclei; mice; ionizing radiation; low dose-rate irradiation; low doses

C. Mothersill et al. (eds.), Multiple Stressors: A Challenge for the Future, 429–438.

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 indicates that low-dose IR exposure induces a complex of biochemical and biophysical reactions in animals (Calabrese and Baldwin, 2000; Mothersill and Seymour, 2003). 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.

In this chapter, the results of the three independent experiments on study of molecular and cellular effects in CBA/lac male mice chronically (up to 1 year) exposed to γ-radiation at a dose rate of 61 cGy/year are reviewed.

Experimental Design

In chronic IR exposure experiments, 4–5-week-old CBA/lac male mice weighting 12–14 g (purchased from "pitomnik-Stolbovaya") were used. Mice were placed in plastic cages 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 2004. Three independent experiments with identical conditions were performed, utilizing totally 420 mice.

Experimental animals were chronically exposed to IR from a γ-ray unit "UOG-1" (VNIIFTRI, Russia) equipped with a ^{137}Cs 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).

To deliver to animals, total cumulative doses of 6.7, 13.4, 20.2, 35.3, 45.4, and 61.3 cGy, low dose-rate irradiation was performed for 40, 80, 120, 210, 270, and 365 days, respectively.

Upon completion of chronic low dose-rate irradiation, mice were sacrificed and spleens were removed and processed for subsequent analysis. 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.

The DNA–protein cross-links level (DPC) was determined by the K^+/SDS assay (Zhitkovich and Costa, 1992) with minor modifications (Osipov et al., 2000). The amount of DNA was measured with the Hoechst 33258 reagent using a FL-2110 fluorimetric analyzer (Solar, Belarus) with excitation 365 nm and emission 460 nm. The level of DPC was determined as a ratio of the amount of DNA in the supernatant to the total DNA in the sample.

Alkali single cell gel electrophoresis was carried out as described by Singh et al. (1988). According to the assay, the number of alkali labile sites and single-strand breaks (SSBs) are proportional to the 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). Hundred comets were counted from each slide. Comets were divided into classes 0–4 (0 corresponded to no visible tail, 4 to 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 (Kobayashi et al., 1995). Results of the visual classification were subsequently confirmed using the analytic package image analysis software (Kinetic Imaging, Liverpool, UK).

A number of comets in each class were recorded and the average comet index (ACI) was calculated as: $ACI = (1 \cdot n1 + 2 \cdot n2 + 3 \cdot n3 + 4 \cdot n4)/\Sigma$, where $n1$–$n4$ are number of comets in classes 1–4 and Σ is the sum of counted comets, including comets in class 0.

Percentage of apoptotic cells was determined by the DNA diffusion assay described elsewhere (Singh, 2000).

For study of the induction of DNA damage in spleen cells by hydrogen peroxide, the cell suspension (1×10^6 cells/mL in PBS) were incubated with H_2O_2 (0.5 and 5 mM) for 30 min at 37°C. The level of DNA strand breaks was estimated by the DNA precipitation assay developed by Olive (1988) and adapted for fluorometric DNA measurement.

Statistical analysis of experimental results was performed using Student *t*-test. The results are presented as mean ± standard error.

Experimental Data

DNA–PROTEIN CROSS-LINKS

DPC are important type of damage in cells, induced by some chemical and physical agents (Oleinick et al., 1987). DPC represent bulky molecular lesions, which are hardly repaired, thereby interfering with normal functioning of the nuclear chromatin and causing serious genetic consequences whenever these lesions fall in noted essential DNA regions.

Figure 1 shows exposure time-dependent changes of DPC level in spleen cells of mice irradiated at a dose rate of 61 cGy/year.

The increased percentage of DNA tightly bound to proteins in irradiated relative to unirradiated control animals are considered to reflect induced by γ-irradiation a formation of DPC. As seen in Fig. 1 temporal (doses) dependencies of DPC levels are nonlinear. The maximum levels of DPC were recorded at day 10 and 30–40 (total doses 1.7, 5.1–6.8 cGy, respectively). The DPC level ratio of experimental to control animals at maximum points was ~1.5–1.7.

It is possible that the mechanism of DNA–protein cross-links formations at low doses of external γ-radiation is nonspecific and reflects the structural rearrangements of chromatin. In this case, topoisomerases may be the proteins involved in the DNA–protein cross-links formations under low dose rate irradiation.

SINGLE-STRAND DNA BREAKS

The comet assay study on spleen cells showed that very low dose-rate irradiation resulted in statistically significant increase in single-strand DNA

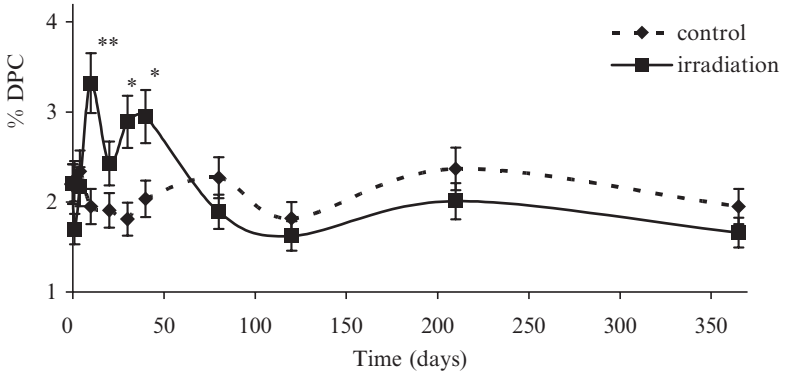

Fig. 1. Exposure time-dependent changes of percentage of DNA tightly bound to proteins (% DPC) in spleen cells of mice exposed to γ-radiation at a dose rate of 61 cGy/year.* – Statistically significant with $P < 0.05$. ** – Statistically significant with $P < 0.01$

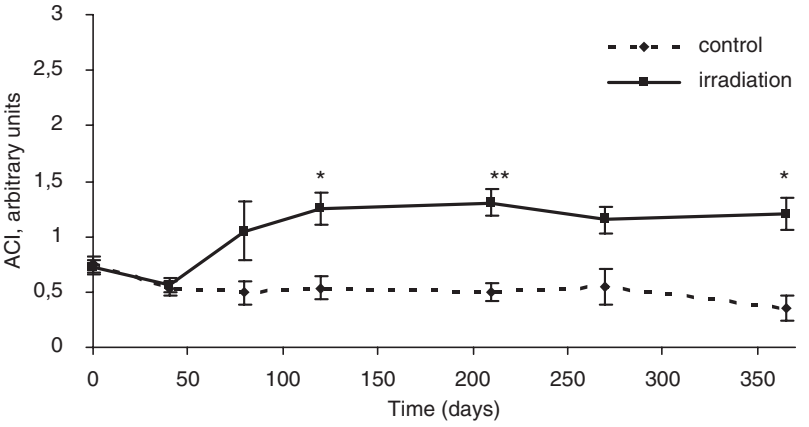

Fig. 2. Chronic irradiation induced time-dependent changes in the single-strand DNA breaks level in mouse spleen cells estimated by alkaline comet assay. ACI – Average comet index. $^{*}P < 0.05$, $^{**}P < 0.01$

breaks level, starting from a dose of 20 cGy (Fig. 2). Further prolongation of exposure time and, hence, increase of a total dose did not, however, lead to further increase in the extent of DNA strand breaks level. A dose–response curve for DNA SSBs is good fitted by a polynomial regression: $y = 0.6209 + 0.0313^{*}x - 0.0004^{*}x^2$, where y is the ACI, x is a dose in cGy.

PERCENT OF APOPTOTIC CELLS

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 twofold increase over a control level in the apoptotic cell fraction was observed (Fig. 3). 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 versus 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).

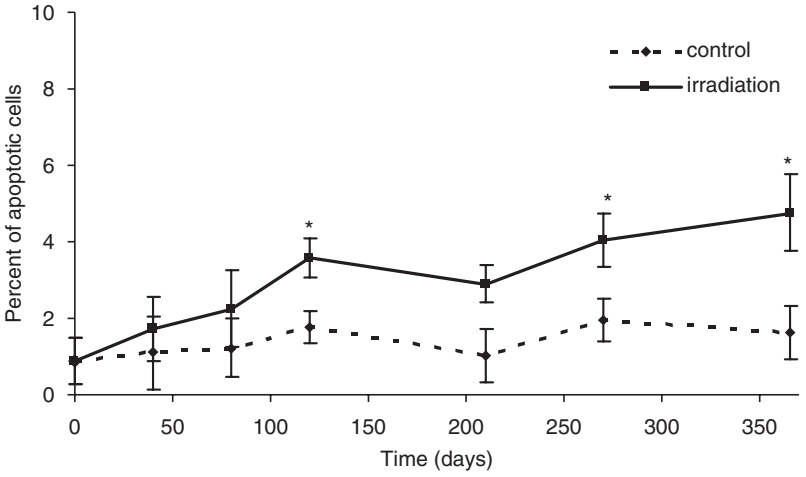

Fig. 3. Percent of apoptotic spleen lymphocytes in control mice and mice exposed to chronic very low dose-rate ionizing radiation for the different times

CELLULAR SENSITIVITY TO ADDITIONAL EXPOSURE

To study the exposure-time changes of the spleen cells sensitivity to additional exposure in the irradiated mice, we have investigated the induction of DNA damage in these cells by hydrogen peroxide. Hydrogen peroxide is a normal cell metabolite which in the presence of redox-active metals (e.g., Fe^{2+}) produced the formation of the highly toxic hydroxyl radical. An increase in sensitivity of the spleen cells from irradiated mice to hydrogen peroxide was revealed at 40 days of experiment (Fig. 4). This fact can probably be explained by the weakening of antioxidant potential/repair capacity in these cells. By 80 days, difference in responses of experimental and control mice splenocytes was decreased. The level of H_2O_2-induced DNA breaks in spleen cells of irradiated animals was slightly higher than that in control cells. Prolongation of irradiation of animals probably activated the defense systems of spleen lymphocytes that were expressed in the decrease of their sensitivity to H_2O_2 exposure at 210 days of experiment (Fig. 4). Thus, our results indicate that chronic action of low dose-rate γ-radiation led to a change in the sensitivity of mice spleen cells to H_2O_2 exposure. A weakening of antioxidant/repair cell potential has been observed at early terms of irradiation. In contrast, prolongation of irradiation resulted in activation of defense system in spleen cells. This effect could be attributed to a development of adaptation processes triggered upon accumulation of a certain dose.

Discussion

An IR dose of 1 Gy induces ~1,000 DNA SSBs per cell (Billen, 1990). Simple calculations based on these data show that irradiation with a dose rate of

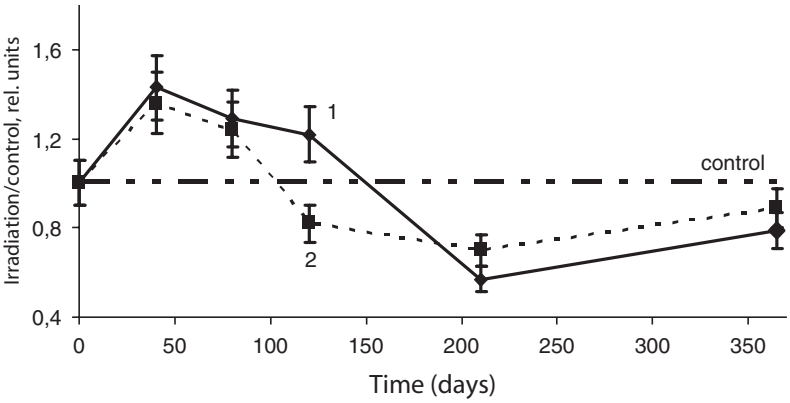

Fig. 4. Dynamics of the sensitivity of the spleen cells from mice continuously exposed to low dose-rate γ-radiation to hydrogen peroxide exposure. Data plotted as the ratio of effects in cells of the experimental animals and that in the control animals

0.07 mGy/h (~0.17 cGy/day) will induce about two DNA SSBs per cell per day, whereas a number of endogenous SSBs resulted from normal oxygen metabolism is ~1.2×10^5 per cell per day (Billen, 1990). 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 the last decade suggest that the particular areas within chromatin possessing hypersensitivity to IR do exist (Oleinick et al., 1994). 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 (Goodhead, 1994). These clusters are thought to be crucial IR-induced DNA lesions leading to double-strand breaks (DSBs), and eventually to mutagenesis and cell death (Ahnstrom and Bryant, 1982). Sutherland et al. (2002) demonstrated that as low as 10 cGy IR caused an increase in clustered DNA damage level in human monocytes. Consistent with our results, nondividing 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 (Rothkamm and Lobrich, 2003).

An indirect mechanism, also known as a «bystander effect», response of unirradiated directly cells due to signaling originating from irradiated cells (Hall, 2003), 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 (Mothersill et al., 2002). 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 (Lorimore and Wright, 2003). A variety of changes has been reported to occur in bystander cells, including overproduction of free radicals (Narayanan et al., 1997; Leach et al., 2001), induction of stress-related kinases, such as JNK, ERK1/2, and others (Little et al., 2002), cytokines β-1-integrin and IL-1α (Osterreicher et al., 2003). 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 (Lehnert and Iyer, 2002). 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 ROS (Chiu et al., 1982; Warters et al., 1987). 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 in active genes is repaired faster and more efficiently compared with that in silent genes (Oleinick et al., 1984; Bohr, 1987).

Our speculation is supported by results of monitoring reparative and replicative DNA syntheses in mouse bone marrow cells reported by Mazurik et al. (2002), 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 earlier, 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 (Mazurik et al., 2002).

It was suggested that the bystander effect has an alternative, protective, feature due to elimination of highly damaged, potentially dangerous cells from a cell population (Belyakov et al., 2002; Prise et al., 2002). In accordance with this line of evidence, an adaptive response, an effect of increased radioresistance to high IR dose acquired after exposure to low doses, was associated with overproduction of ROS (Lehnert and Iyer, 2002). We demonstrated that increase in DNA breaks level by the days 120–365 of low-level IR exposure is accompanied by elevated resistance to hydrogen peroxide treatment. It is possible that the elevated cell resistance could be explained by either activation of DNA repair or elimination of a supersensitive cell population.

Thus, overall increase in the level of DNA breaks in mouse spleen lymphocytes as a result of chronic low dose-rate IR exposure can be possibly explained by structural rearrangement of the chromatin during gene expression activation, free-radical overproduction, and DNA repair activation. Although insignificant, a contribution of apoptotic cells to an overall level of DNA damage was also recorded, providing further support for the proposed mechanisms of low dose-rate radiation-induced effects observed in this study.

The experimental data analysis allows the stage mechanism of the cellular response to chronic low dose-rate ionizing radiation exposure developing with an increase in the exposure time (dose) of irradiation. First stage (doses up to 10–20 cGy) – accumulation of DNA damages (in particularly DNA–protein cross-links) in nonactive (bulk) chromatin, an increase in the cellular sensitivity to additional exposure; second stage (doses of 0.2–0.5 (0.6) Gy) – active response of cells to the damages and as consequence an increase in the quantity of DNA strand breaks, caused by activation of transcription and DNA repair, overproduction of the ROS, and apoptosis induction; acquisitive of the cellular resistance to additional exposure; balance between the DNA damages formation and their repair. And last (hypothetic) third stage (doses upper of 0.5 (0.6) Gy) – additional formation of DNA damages by free radicals due to chromatin conformation changes and exhausting free-radicals defense systems can lead to an increase in the cytogenetic disturbances frequency.

References

Ahnstrom G., and Bryant P.E., 1982, DNA double-strand breaks generated by the repair of X-ray damage in Chinese hamster cells, *Int. J. Radiat. Biol*. 41(6):671–676.

Belyakov O.V., Folkard M., Mothersill C., Prise K.M., and Michael B.D., 2002, Bystander-induced apoptosis and premature differentiation in primary urothelial explants after charged particle microbeam irradiation, *Radiat. Prot. Dosimetry* 99(1–4):249–251.

Billen D., 1990, Spontaneous DNA damage and its significance for the "negligible dose" controversy in radiation protection, *Radiat. Res*. 124:242–245.

Bohr V.A., 1987, Preferential DNA repair in active genes, *Dan. Med. Bull*. 34(6):309–320.

Calabrese E.J., and Baldwin L.A., 2000, Radiation hormesis: its historical foundations as a biological hypothesis, *Hum. Exp. Toxicol*. 19:41–75.

Chiu S.M., Oleinick N.L., Friedman L.R., and Stambrook P.J., 1982, Hypersensitivity of DNA in transcriptionally active chromatin to ionizing radiation, *Biochim. Biophys. Acta* 699(1):15–21.

Goodhead D.T., 1994, Initial events in the cellular effects of ionizing radiation: clustered damage in DNA, *Int. J. Rad. Biol*. 65:7–17.

Hall E.J., 2003, The bystander effect, *Health Phys*. 85(1):31–35.

Kobayashi H., Sugiyama C., Morikawa Y., Hayashi M., and Sofuni T., 1995, A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis assay, *MMS Commun*. 3:103–115.

Leach, J.K., Van Tuyle, G., Lin P.S., Schmidt-Uurich, R., and Mikkelsen, R.B., 2001, *Cancer Res*. 61:3894–3901.

Lehnert B.E., and Iyer R., 2002, Exposure to low-level chemicals and ionizing radiation: reactive oxygen species and cellular pathways, *Hum. Exp. Toxicol*. 21(2):65–69.

Little J.B., Azzam E.I., de Toledo S.M., and Nagasawa H., 2002, Bystander effects: intercellular transmission of radiation damage signals, *Radiat. Prot. Dosimetry* 99(1–4):159–162.

Lorimore S.A., and Wright E.G., 2003, Radiation-induced genomic instability and bystander effects: related inflammatory-type responses to radiation-induced stress and injury? A review. *Int. J. Radiat. Biol*. 79(1):15–25.

Mazurik V.K., Mikhailov V.F., and Ushenkova L.N., 2002, Dynamic component of maintaining genomic stability in murine bone marrow cells after chronic low-intensity irradiation lasting one year, *Radiat. Biol. Radioecol*. 42(6):669–674.

Mothersill C., O'Malley K., and Seymour C.B., 2002, Characterisation of a bystander effect induced in human tissue explant cultures by low let radiation, *Radiat. Prot. Dosimetry* 99(1–4):163–167.

Mothersill C., and Seymour C., 2003, Low-dose radiation effects: experimental hematology and the changing paradigm. *Exp. Hematol*. 31(6):437–445.

Narayanan, P.K., Goodwin, E.Hr., and Lehnert, B.E., 1997, Alpha particles initiate biological production of superoxide anions & hydrogen peroxide in human cells. *Cancer Res*. 57:3963–3971.

Oleinick N.L., Balasubramaniam U., Xue L., and Chiu S., 1994, Nuclear structure and the microdistribution of radiation damage in DNA, *Int. J. Radiat. Biol*. 66:523–529.

Oleinick N.L., Chiu S.M., and Friedman L.R., 1984, Gamma radiation as a probe of chromatin structure: damage to and repair of active chromatin in the metaphase chromosome, *Radiat. Res*. 98(3):629–641.

Oleinick N.L., Chiu S.-M., Ramakrishnan N., and Xue L.-Y., 1987, The formation, identification, and significance of DNA–protein cross-links in mammalian cells, *Brit. J. Cancer* 55:135–140.

Olive, P.L., 1988, DNA precepitation assay: a Rapid & Simple method for detecting DNA damage in mammalian cells, *Environ. Mol. Mutagen* 11:487–495.

Osipov A.N., Sypin V.D., Puchkov P.V., Razumova A.S., and Kuznetsova E.M., 2000, Changes in the level of DNA–protein cross-links in spleen lymphocytes of mice exposed to low-intensity γ-radiation at low doses, *Radiat. Biol. Radioecol*. 40(5):516–519.

Osterreicher J., Skopek J., Jahns J., Hildebrandt G., Psutka J., Vilasova Z., Tanner J.M., Vogt J., and Butz T., 2003, Beta1-integrin and IL-1alpha expression as bystander effect of medium from irradiated cells: the pilot study, *Acta Histochem*. 105(3):223–230.

Prise K.M., Belyakov O.V., Newman H.C., Patel S., Schettino G., Folkard M., and Michael B.D., 2002, Non-targeted effects of radiation: bystander responses in cell and tissue models, *Radiat. Prot. Dosimetry* 99(1–4):223–226.

Rothkamm K., and Lobrich M., 2003, Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses, *Proc. Natl. Acad. Sci. USA* 100(9):5057–5062.

Singh N.P., 2000, A simple method for accurate estimation of apoptotic cells, *Exp. Cell Res*. 256(1):328–337.

Singh N.P., McCoy M.T., Tice R.R., and Schneider E.L., 1988, A simple technique for quantification of low levels of DNA damage in individual cells, *Exp. Cell Res*. 175:184–191.

Sutherland B.M., Bennett P.V., Cintron-Torres N., Hada M., Trunk J., Monteleone D., Sutherland J.C., Laval J., Stanislaus M., and Gewirtz A., 2002, Clustered DNA damages induced in human hematopoietic cells by low doses of ionizing radiation, *J. Radiat. Res*. (*Tokyo*) 43(Suppl):149–152.

Warters R.L., Lyons B.W., Chiu S.M., and Oleinick N.L., 1987, Induction of DNA strand breaks in transcriptionally active DNA sequences of mouse cells by low doses of ionizing radiation, *Mutat. Res*. 180(1):21–29.

Zhitkovich A., and Costa M., 1992, A simple, sensitive assay to detect DNA–protein cross-links in intact cells and in vivo, *Carcinogenesis* 13:1485–1489.