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Dose- and time-dependent changes of micronucleus frequency and gene expression in the progeny of irradiated cells: Two components in radiation-induced genomic instability?

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ABSTRACT

Murine embryonic C3H/10T½ fibroblasts were exposed to X-rays at doses of 0.2, 0.5, 1, 2 or 5 Gy. To follow the development of radiation-induced genomic instability (RIGI), the frequency of micronuclei was measured with flow cytometry at 2 days after exposure and in the progeny of the irradiated cells at 8 and 15 days after exposure. Gene expression was measured at the same points in time by PCR arrays profiling the expression of 84 cancer-relevant genes. The micronucleus results showed a gradual decrease in the slope of the dose–response curve between days 2 and 15. The data were consistent with a model assuming two components in RIGI. The first component is characterized by dose-dependent increase in micronuclei. It may persist more than ten cell generations depending on dose, but eventually disappears. The second component is more persistent and independent of dose above a threshold higher than 0.2 Gy. Gene expression analysis 2 days after irradiation at 5 Gy showed consistent changes in genes that typically respond to DNA damage. However, the consistency of changes decreased with time, suggesting that non-specificity and increased heterogeneity of gene expression are characteristic to the second, more persistent component of RIGI.

1. Introduction

The classical paradigm of radiobiology assumed that direct radiation-induced DNA damage is the main cause of the biological effects of ionizing radiation. Such direct damage, if not correctly repaired, was assumed to result in cell death or heritable changes that are responsible for the late effects (such as cancer) in the progeny of the irradiated cells. However, numerous studies have shown that ionizing radiation is able to induce a state of genomic instability in cultured cells. Radiation-induced genomic instability (RIGI) is a concept used to describe the finding that delayed damage (chromosomal aberrations, mutations, micronuclei, apoptosis) is observed many cell generations later in the progeny of irradiated cells (for reviews, see [1–4]). This finding indicates that cells inherit not only direct mutations but an increased frequency of genetic damage. As the development of cancer requires accumulation of multiple genetic changes, RIGI is believed to be relevant to cancer [5–7], and also transgenerational RIGI in animals and humans has been described [8,9]. The target size for RIGI is large, and the effect cannot be due to mutations in a single gene, or even a group of genes [1,3,10]. This indicates an epigenetic effect, but the mechanisms remain unclear [11].

The results of some studies suggest that the dose–response relationship for RIGI is flat (with no or little dependence on dose) above a threshold [12], but the data are partly inconsistent [13]. Time-dependent changes after irradiation may explain some of the inconsistencies, but evaluating this hypothesis is difficult, as only a few studies have investigated dose–response at more than one point in time.

Studying gene expression changes in the exposed cells and their progeny might be useful for understanding the biological processes that lead to RIGI. Immediate (up to 48 h after irradiation) transcriptional responses to radiation-induced DNA damage have been investigated in many studies, and the findings naturally

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include changes in the expression of genes involved in DNA damage response [14–17]. However, limited data are available concerning gene expression changes at delayed endpoints, corresponding to development of RIGI. Mello et al. [18] reported changes in the transcription profile of human primary fibroblasts exposed to 0.5 Gy of gamma rays, but the analysis was done at only one point in time (6 days). Two studies have analyzed gene expression changes at several time points after irradiation. Falt et al. [34] studied gene expression patterns in cultured human lymphocytes grown for 7, 17 or 55 days after exposure to 3 Gy of gamma rays. Kruse et al. [19] induced kidney damage in mice by *in vivo* exposure to X-rays at 16 Gy and performed microarray analysis of gene expression in kidney tissue at 1, 10, 20 and 30 days after exposure. Both studies reported gene expression profiles that changed strongly over time, with very few genes consistently up- or downregulated at all time points studied. Falt et al. [34] also reported a more diversified pattern of gene expression changes in the irradiated than control cells, suggesting that increased heterogeneity of gene expression might be characteristic of RIGI. However, the study did not include simultaneous measurement of any other endpoints than gene expression to confirm RIGI by independent methods and to follow the co-development of RIGI and gene expression changes.

The aim of the present study was to investigate the dose–response for both direct effects (2 days after irradiation) and RIGI assessed at two delayed points in time, under the hypothesis that the dose–response changes with time. Furthermore, gene expression was analyzed at the same points in time to relate gene expression changes to the development of RIGI. Gene expression was examined both to identify possible changes specific to RIGI and to test the hypothesis that the development of RIGI is associated with increased heterogeneity of gene expression. A wide dose range from 0.2 to 5 Gy was used to include the high doses used in many previous studies. Measurement of micronuclei (MN) was used for monitoring the development of RIGI. Micronuclei are an easily detectable and widely used indicator of different types of direct chromosomal injuries, but they have also been found to be constantly elevated over multiple generations in the progeny of irradiated cells [20] and they have been therefore used for detecting genomic instability induced by ionizing radiation as well as other exposures in a variety of cells types [21–23]. Gene expression was measured by PCR arrays, which allow profiling only a limited number of genes but provide highly reproducible data. As RIGI is assumed to have a role in radiation carcinogenesis, a PCR array profiling cancer-relevant genes was selected.

2. Materials and methods

2.1. Cell line and cell culture

Murine embryonic fibroblasts (C3H/10T½ clone 8) from American Type Culture Collection (Manassas, VA, USA) were used, as we have previously shown chemically induced genomic instability in the same cell line [24]. The cells were maintained in Eagle's basal medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) (all from Gibco, Carlsbad, USA). The cells were grown at 37 °C with 5% CO_2 and detached by incubating them for 5 min in 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) in 0.02% EDTA in PBS (w/o Ca^{2+}, Mg^{2+}), after which the trypsin was inactivated by adding a double volume of fresh +37 °C medium. The subcultured cell density was selected depending on the X-ray dose, end-point to be analyzed and time point of the analysis, assuming a doubling time of 24 h (the actual doubling time of this cell line is approximately 16 h with some delay after the plating [25]).

In order to get 400,000 cells for the micronucleus assay conducted 2 days after the exposure, cells were seeded on 60 mm plates at a density of 2300 cells/cm^2 24 h prior to the exposure. For the cells to be collected 8 days after the exposure, the following cell densities (based on previous viability experiments) were plated on T75 flasks 24 h before the exposure: 200 cells/cm^2 (controls and 0.2 Gy, 0.5 Gy and 1 Gy), 270 cells/cm^2 (2 Gy), and 400 cells/cm^2 (5 Gy). Six days after the exposure cells were counted again and seeded on plates at a density of 4700 cells/cm^2. At this same time point plates with 1000 cells on each were seeded for the micronucleus analysis 15 days after the exposure.

For gene expression analysis 2 days after exposure, 2700 cells/cm^2 (control, and 1 Gy), or 4500 cells/cm^2 (5 Gy) were plated on T75 flasks 24 h before the exposure. For the same analysis to be conducted 8 days after exposure, 330 cells/cm^2 (controls, and 1 Gy) or 670 cells/cm^2 (5 Gy) were plated on T75 flasks 24 h prior exposure. 5 days after the exposure these cells were subcultured on T75 flasks at the density of 2700 cells/cm^2 (controls, and 1 Gy) or 4500 cells/cm^2 (5 Gy). The cells to be analyzed 15 days after exposure were also subcultured on T75 flasks at 5 days; the cell density was 330 cells/cm^2 (controls, and 1 Gy) or 530 cells/cm^2 (5 Gy).

Viability and proliferation assay was performed using AlamarBlue reagent (Bio-Source International, Inc., Camarillo, CA, USA) according to the manufacturer's protocol. Briefly, cells were washed twice with ice-cold PBS (phosphate buffered saline without Ca^{2+}, Mg^{2+}, Oy Reagena Ltd, Toivala, Finland), removed from plates by scraping in 1 ml of ice-cold PBS. Cells were counted and transferred in 450 μl of cell culture medium into 96-well plates (Nunc, Roskilde, Denmark) 24 h before irradiation at cell densities 2000 cells/well (0.5 Gy), 4000 cells/well (1 Gy), or 8000 cells/well (2 and 5 Gy). Control well plates were made using all the cell densities used in the exposures. Then 50 μl of AlamarBlue was added to each well. Culture medium was used as blank samples. Fluorescence was measured immediately after the 96-well plates were ready, 1 h before exposure, 2 h after exposure, 12 h after exposure and once in every 24 h for the 7 days following the exposure. The plates were exposed to an excitation wavelength of 540 nm and the emission at 595 nm was recorded on a fluorometer (Perkin Elmer HTS 7000 Plus Bio Assay Reader, Turku, Finland). The viability was expressed as a ratio of fluorescence emitted by treated cells compared to control cells.

2.2. Exposure

The cells were exposed to X-rays with a 4 MV Varian 600 C linear accelerator (Varian, Palo Alto, CA, USA) at the Kuopio University Hospital. The cell cultures were placed on the treatment couch between two plexiglass sheets 1 cm above and 2 cm below the samples. The control cultures were also taken into the hospital facilities. Doses of 0.2, 0.5, 1, 2 and 5 Gy were used. The maximum photon energy was 4 MeV (average 1.5 MeV) and the dose rate about 2.5 Gy/min.

2.3. Micronucleus assay

The frequency of micronuclei was measured by a flow cytometric assay as described previously [26]. The principle of the assay is that the nuclei of dying cells are stained with ethidium monoazide bromide (EMA), followed by lysis of the viable cells and staining of their nuclei with SYTOX Green dye. Finally, nuclei are assorted by flow cytometer.

In brief, medium was removed from the plates containing cell cultures and the plates were incubated on ice for 20 min. After the incubation, 1.5 ml of 8.5 μl/ml +4 °C EMA-solution (Molecular Probes, Eugene, USA) was added and the stain was light-activated for 30 min with a light bulb 15 cm above the plates (lids removed).