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Metformin prolongs survival rate in mice and causes increased excretion of cell-free DNA in the urine of X-irradiated rats

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ABSTRACT

An antidiabetic drug metformin has anticarcinogenic and geroprotective effects and has been used in combination with radiation cancer therapy. The present work is devoted to the study of the effect of metformin on survival in mice, the frequency of micronuclei in mouse bone marrow cells and excretion of cell-free nuclear and mitochondrial DNA in the urine of X-ray-exposed rats. The survival rate and the frequency of micronuclei in mice and excretion of DNA into rat urine were determined after administration of the drug before and after irradiation of animals. The DNA content was measured by qRT-PCR. Metformin shows a radioprotective effect only when administered to mice after the radiation exposure. On the 11th day after irradiation, we observed 100% mortality in the control group; 78% of mice remained alive if metformin was given. Twenty percent of the mice in this group survived for 30 days after irradiation. Metformin has the same effect on the frequency of micronuclei; its reduction is observed, when the drug is administered to the mice after irradiation. Metformin promotes the excretion of nuclear and mitochondrial DNA with the urine of irradiated rats. The results show that metformin acts as a radiomitigation effector. Metformin promotes the active excretion of DNA of dying cells from the tissues of irradiated animals.

1. Introduction

The death of cells with structural and functional abnormalities in the tissues of animals and humans is an active process that supports the renewal of cell populations, functional integrity and homeostasis of tissues. An increase of cell-free DNA (cf-DNA) in biological fluids can be seen as a result of cell death activation in the tissues under the influence of exogenous and endogenous factors and it is used as a biomarker for diagnosis and prognosis of different diseases, particularly in treatment of tumors [1,2]. Different modulators of cell death also help to assess changes in the amount of cf-DNA in biological liquids of animals and humans. These modulators include *N, N'*-dimethyl biguanide [Metformin (MF)], which has been used as a first-line drug in the treatment of type 2 diabetes for many years, though the mechanism of action of metformin remains poorly understood and controversial [3]. However, MF is not only a medication used to treat diabetes mellitus type 2, but it is also known as an anticarcinogen and geroprotector [4,5]. Also, MF makes radiation therapy more effective when used for the patients suffering from cancer in combination drug treatment [6]. Earlier we have demonstrated that excretion of the fragments of nuclear DNA (cf-nDNA) and cf-mitochondrial DNA (cf-mtDNA) into urine of X-irradiated rats increased significantly, thereby indicating the activation of radiation-induced cell death signaling pathway [7]. Moreover, 24-month old rats exposed to ionizing irradiation (IR) demonstrate a more pronounced increase in the cf-nDNA and cf-mtDNA levels in the urine compared to those in the urine of young rats [8]. These findings may let us suppose that MF facilitates the removal of the cells and organelles with structural and functional abnormalities that originated from irradiation-induced damage or accumulated with age. Removal of damaged cells and malfunctioning organelles from the tissues by activation of the mechanisms of apoptosis and autophagy can maintain health under stress-related conditions.

In this work we studied the effect of MF on survival of mice and on micronuclei formation (MN) in the cells of bone marrow after X-ray irradiation and also investigated the influence of MF on excretion of cf-nDNA and cf-mtDNA into urine of irradiated rats.

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2. Materials and methods

2.1. Animals and their treatment

Male Balb/c mice aged 2 months and Fisher-344 male rats aged 3 months were obtained from the Animals Breeding Center of the Branch of Institute of Bioorganic and Chemistry, Russian Academy of Sciences (Pushchino, Moscow Region). All experiments with animals followed the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, Directive 2010/63/EU. Mice and rats were fed a special diet for mice and rats ad libitum, with free access to clean drinking water ad libitum. The animals were housed 5 mice or 3 rats per cage, in a standard 12-h light/12-h dark cycle at a temperature of 22 ± 2 °C and 45 ± 5% humidity.

The animals were irradiated at the Common Use Centre – Group of Radiation Sources of the Institute of Cell Biophysics RAS, using an X-ray apparatus RTU-12 (280 kVp, 20 mA) with 1 mm AL and Cu filters at a dose rate of 1 Gy/min. Mice (5 animals) and rats (3 animals) were irradiated in plastic containers. Mice were irradiated at a dose of 8 Gy to define their survival rate [9] or at a dose of 2 Gy to assess the formation of micronuclei in bone marrow cells [10]. Rats were irradiated at a dose of 5 Gy [7].

MF (Merck, Darmstadt, Germany) was given perorally to animals at a dose of 300 mg/kg body weight in drinking water using a plastic pipette. At this dose MF does not exert a genotoxic effect and even mice administered in daily doses of 600 mg/kg body weight for 9 months have not shown significant kidney failure [11]. Mice received MF 25 min before or 15 min, 6 and 24 h after the radiation exposure. Animal survival was monitored for 30 days after irradiation, and the number of mice that survived was checked at the same time every day. In 5 groups of mice, each included 30 animals for determination of the survival rate. Rats wereadministered MF in 15 min and 24 h after irradiation. In these experiments each group composed of 8 rats.

2.2. Micronucleus test

Cytogenetic test of micronuclei (MN) in bone marrow cells of mice was carried out using standard methods [12]. Mice were irradiated at a dose of 2 Gy and MF was administered as mentioned before orally 25 min prior to irradiation and in 15 min, 6 and 24 h after irradiation. Non-irradiated mice with and without MF administration were used as the control group. The mice were killed by cervical dislocation 28 h after irradiation. Each group included 8 mice. Their femoral bone marrow was flushed out with fetal calf serum, and cell suspension was prepared. The suspension was centrifuged for 7 min at 1000 rpm. After centrifuging, the supernatant was removed, the cells were re-suspended in serum, and a smear was prepared, fixed with methanol and stained in May–Grunwald–Giemsa (Merck, Darmstadt, Germany). With this method of staining, polychromatic erythrocytes (PCE) are stained blue-violet, while normochromatic erythrocytes (NCEs) are stained yellow-orange. For each mouse 4 slides were prepared, in total 2000 polychromatic erythrocytes were counted to determine the frequency of MNPCE.

2.3. Urine collection and DNA extraction

In each case the urine was collected from the same mice before irradiation and MF administration (controls) and after irradiation and MF administration. The urine was collected at different time points after irradiation and administration of MF. In one group mice were administered MF in 24 h after irradiation and the obtained results were compared to the data from irradiated rats which were not given MF. For urine collection rats were placed in individual metabolic cages (Hatteras Instruments, USA) equipped with glass containers. To the bottom of the container 0.5 ml of 0.1 M EDTA (pH 8.0) solution was added and covered with a layer of paraffin oil. Urine was collected in the evening starting at 6 p.m. at 22 ± 2 °C during 5.5–6 h until there was 5 ml of liquid collected in the container. Urine samples were centrifuged (5000 r/min, 10 min) and the supernatant was frozen at −20 °C for 20–24 h before DNA extraction. Frozen urine samples were thawed at room temperature and then placed on ice prior to total DNA extraction (mtDNA and nDNA). DNA was extracted using Wizard Plus Minipreps DNA Purification System (Promega, USA) in accordance with the manufacturer's instructions and as described [13]. Each DNA sample was dissolved in 0.1 ml of Millipore Q water. The concentration of DNA was determined by reaction with Pico Green reagent according to the manufacturer's protocol (Molecular Probes, Eugene, USA), followed by fluorescence detection on Tecan Infinite (Austria). To estimate the length of DNA fragments, total DNA samples were subjected to electrophoresis in 1% agarose gel stained with ethidium bromide. Electrophoresis was carried out using Bio-Rad (USA) equipment as previously described [7].

2.4. Quantitative analysis of nuclear and mitochondrial DNA by real time PCR method

Quantitative analysis of nDNA and mtDNA was carried out by real-time PCR with TaqMan oligonucleotides on a Prism 7500 thermal cycler (Applied Biosystems, USA). The changes in the relative quantity of mtDNA and nDNA copies were determined as a ratio between the number of copies of the mitochondrial tRNA gene and that of GAPDH gene of nDNA in the same test tube. The efficiency of PCR for amplifying both nDNA and mtDNA was measured using standard curves generated for a dilution series with 20, 10, 5, 2, 1 and 0.1 ng of total rat liver DNA per reaction. As the basis for nDNA and mtDNA copies quantification we used the threshold level of cycle (Ct) [14]. PCR tests were carried out in triplicate for each DNA sample. The following primers were used for tRNA gene amplification (73 bp) from mtDNA: forward 5′- AAT GGT TCG TTT GTT CAA CGA TT −3′; reverse 5′- AGA AAC CGA CCT GGA TTG CTC −3′; and a probe − R6G-AAG TCC TAC GTG ATC TGA GTT-RHQ1. For GAPDH gene amplification (80 bp) from nDNA the following primers were used: forward 5′- TGG CCT CCA AGG AGT AAG AAA C −3′; reverse 5′- GGC TCT CTC CTT GCT CTC AGT ATC −3′; and a probe – FAM-CTG GAC CAC CCA GCC CAG CAA-RTQ1. Primers and probes for mtDNA and nDNA were chosen using BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), in the sequence that excludes co-amplification of NUMT-pseudogenes in nDNA [15]. The following PCR program was used: 5 min at 95 °C followed by 40 cycles (95 °C for 30 s, annealing and elongation at 60 °C for 1 min).

2.5. Statistical analysis

Statistical differences between the data obtained before and after treatment of rats were estimated by the Mann-Whitney *U* test or the standard Student's unpaired *t*-test when appropriate. In the survival experiments, the survival curves of different groups were compared by Fisher's exact test. The results were calculated and presented as a mean ± SE of the mean (SEM, n = 8). P value < 0.05 was considered to be statistically significant.

3. Results

3.1. Survival of mice exposed to a lethal dose of X-rays

Fig. 1 shows the survival curves for mice exposed to X-irradiation at a lethal dose of 8 Gy. These data show that mice (100%) were found dead on the 11th day after irradiation in the control group. Survival results was same in the group of mice which were administered MF 25 min before irradiation. However, in the groups of mice which were given MF in 6 h and 24 h after irradiation, a significant delay in time to death was registered. Thus, in the group of animals with MF administration in 6 h after irradiation the number of animals that survived was