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Electrophysiologic and cellular characteristics of cardiomyocytes after X-ray irradiation

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ABSTRACT

The aim of this study was to investigate possible effects of ionizing irradiation on the electrophysiological functionality of cardiac myocytes in vitro. Primary chicken cardiomyocytes with spontaneous beating activity were irradiated with X-rays (dose range of 0.5–7 Gy). Functional alterations of cardiac cell cultures were evaluated up to 7 days after irradiation using microelectrode arrays. As examined endpoints, cell proliferation, apoptosis, reactive oxygen species (ROS) and DNA damage were evaluated. The beat rate of the cardiac networks increased in a dose-dependent manner over one week. The duration of single action potentials was slightly shortened. Additionally, we observed lower numbers of mitotic and S-phase cells at certain time points after irradiation. Also, the number of cells with γH2AX foci increased as a function of the dose. No significant changes in the level of ROS were detected. Induction of apoptosis was generally negligibly low.

This is the first report to directly show alterations in cardiac electrophysiology caused by ionizing radiation, which were detectable up to one week after irradiation.

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1. Introduction

Ionizing radiation (IR) is a recognized risk factor for cardiovascular diseases. Several studies have shown an increased risk of heart stroke and comparable diseases in patients after radiotherapy for cancer [1,2]. Epidemiological studies point to a risk of adverse cardiovascular effects even at low and moderate doses of IR, typically developing with a long latency. Cardiac dysfunction is suggested to be an indirect effect following radiation-induced vascular damage [3]. In vivo studies showed that a dose of 10 Gy X-rays increases the risk of coronary sclerosis and heart degeneration in rats [4], while cardiomyocytes maintained normal appearance. Proteomic studies of neonatal mouse hearts irradiated with doses of up to 1 Gy gamma-rays point toward a long-term deregulation of proteins involved in metabolic processes, inflammatory response and cytoskeletal structure [5]. Seemann et al. [6] found that irradiation with 2 and 8 Gy induced modest changes in murine cardiac function as well as progressive structural and microvascular damage. In a subsequent study they found multiple genes responsible for the maintenance and protection of cardiomyocyte functions to be upregulated in the hearts of mice irradiated with 16 Gy X-rays [7].

Very recently, in vitro evidence was adduced that IR affects calcium handling in cardiac myocytes by elevated ROS levels [8]. Sag et al. concluded that this cardiac dysfunction stems from radiation-induced calcium handling impairment caused by increased oxidative stress and persistently activated calcium/calmodulin-dependent protein kinase II. Dysfunctions of the cell-to-cell communication may result in arrhythmia and, in the worst case, lead to lethal ailments such as *Torsade de pointe*, sudden cardiac death or congestive heart failure. Furthermore the study by Bakhsi et al. [5] showed that cell-to-cell signaling and cellular interaction are most commonly affected in gamma irradiated mouse hearts regardless of dose. This evidence in mind, we hypothesize that IR

Abbreviations: IR, ionizing radiation; MEA, microelectrode array; fAPd, field action potential duration; TUNEL, TdT-mediated dUTP nick end labeling; GSH, monormeric glutathione; GSSG, dimeric glutathione; DTNB, Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid); ESA, European Space Agency; DLR, German Aerospace Agency; IBER, Investigations of Biological Effects of Space Radiation; PTM, post-translational modification.

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could induce changes in the electrophysiological functionality of cardiac myocytes.

Cardiac electrophysiological assays are widely used in pharmacological studies. In radiation biology, however, up to now electrophysiological applications are rarely employed. Therefore, little is known about potential direct effects of irradiation on electrophysiology of cardiac myocytes on the cellular level. The most commonly applied techniques such as the Langendorff heart, or the patch clamp assay [9], offer insights into the whole organ, or on the level of single cells. The microelectrode array (MEA) technology in contrast is a non-invasive method which can not only be used to analyze action potential rate and shape, but can further detect and analyze the propagation of ion current signals across the cardiac syncytium at the tissue level.

In the present study we applied the MEA technology to investigate the effects of X-rays on electrically active cardiac cells. The selected doses ranged from 0.5 Gy to 7 Gy, as we suspected from recent studies that lower doses show hardly discernable electrophysiological changes. While higher doses above 7 Gy on the other hand proofed to be non-fatal in preliminary experiments (Frieß et al., unpublished data) and are furthermore not relevant for clinical applications. Primary cardiac myocytes isolated from chicken embryos were utilized, as this model system allows a higher cell yield compared to mammalian cells. Furthermore, preceding studies have shown that cell cycle regulation as well as DNA damage repair regulation in chicken is similar to mammalian cell lines [10–13]. Also chicken eggs can be reliably bred to the desired developmental stage, and the hearts of embryos are easier to obtain than in mammalian model systems. Here, for the first time, systematic investigations of radiation effects on the electrophysiology of cardiac myocytes were performed, using the benefits of MEA chips. In addition, experiments were conducted to examine the effect of X-rays on the cell cycle propagation, apoptosis, DNA-damage repair and oxidative stress in cardiomyocytes.

2. Material and methods

2.1. Cardiomyocyte cultivation and irradiation

White leghorn chick embryos were harvested on day 8 after fertilization (E8). Embryos were decapitated and hearts were collected in Ham's F12 (Sigma, Taufkirchen, Germany) and processed as described by Nick et al. [14]. Briefly, coronary vessels were removed and blood cells rinsed out with Ham's F12. The hearts were minced in 1 mL *Hanks balanced salt solution* (HBSS; Sigma, Taufkirchen, Germany) using microscissors. Cut tissue was collected in a 15 mL centrifuge tube and washed with HBSS. The tissue was then enzymatically dissociated as follows: For the first cell fraction 8 mL 1 × trypsin/EDTA (Sigma, Taufkirchen, Germany) were added and cell suspension was gently inverted for 5 min, followed by incubation for 5 min at 37 °C. The supernatant of the first fraction, containing mainly fibroblasts, was discarded. For the following cell fractions 3.5 mL 1 × trypsin/EDTA were added, suspension gently inverted for 4 min, followed by incubation for 4 min at 37 °C. The supernatant was collected in 15 mL pre-warmed culture medium (DMEM, 10% fetal calf serum, 2% chick serum, 2 mM L-glutamine, 0.1% penicillin/streptomycin; all Sigma, Taufkirchen, Germany). The procedure was repeated until the tissue was completely dissociated. To remove cell clusters, the collected cell suspension was filtered with a cell strainer (0.4 μm; BD Biosciences, Heidelberg, Germany) and centrifuged. The pellet was resuspended in 13 mL culture medium. To reduce the number of fibroblasts the cell suspension was seeded in a cell culture flask (Sigma, Taufkirchen, Germany) and incubated for 1 h at 37 °C and 5.0% CO_2. Then, the medium, containing mainly cardiomyocytes was removed, cell titer determined and seeded on fibronectin-coated (50 μg/mL; Sigma, Taufkirchen, Germany) MEA chips or glass cover slips, at defined densities of 600,000 cells in a 100 μL droplet or 200,000 cells/mL respectively. A complete removal of fibroblasts is not desired as the functionality of primary cardiomyocytes requires fibroblasts [15]. Irradiation with X-rays (90 kV, 33.7 mA, 5.23 Gy/min) was performed in culture medium at room temperature; thereby we considered that cells X-irradiated on glass slides receive a higher dose as determined by physical and chemical dosimetry [16]. All controls were sham irradiated.

2.2. Electrophysiology

The electrical activity of the cardiac cells developing a beating syncytium was measured, using the non-invasive extracellular recording methods of microelectrode array chips (MEA; Multichannel Systems, Reutlingen, Germany). The 60 TiN microelectrodes with a diameter of 30 μm are embedded in a glass substrate and arranged in an 8 × 8 array with an interelectrode distance of 200 μm. Measurements were performed using a LabView-programmed software at a sampling rate of 1 kHz. Data evaluation was conducted offline, applying the MATLAB-based software tool *DrCell* [17]. Examined parameters were the number of active electrodes, beat rate, conduction velocity, field action potential duration (fAPd) and positive and negative amplitudes. Medium exchange was performed daily at least 60 min prior to the measurements. During measurements, cultures were kept at a constant temperature of 37 °C.

The cells were analyzed as early as possible (i.e. 30 min after exposure). Intervals were chosen according to the procedure demands. Since cells detached after 7–9 days regardless of received dose, the last analysis was performed 7 days after irradiation. After irradiation, measurements were performed twice per day with a 3 h interval. The data were averaged in order to obtain more robust mean values per day.

2.3. Immunohistochemistry

For S-phase detection, cells were incubated with 25 μM BrdU for 30 min (for detection 30 min after irradiation) or 1 h (for later time points) prior to fixation. Samples were fixed in methanol/acetate (19:1), followed by washing in PBS buffer and incubation in 2 N HCl for 10 min, and then neutralized in PBS prior to antibody staining. For all other endpoints cells were fixed for 10 min in 4% paraformaldehyde, followed by washing with PBS buffer. Immunohistochemical staining was usually performed by incubation with primary antibody (BrdU: 1:500, G3G4 (DSHB, Iowa City, IO, USA); γH2AX: 1:500 anti-phospho-histone H2AX (Ser139) antibody (Millipore, Billerica, MA, USA) anti-phospho-histone H3 antibody: 1:200, pH3, (Santa Cruz, TX, USA)) in 0.1% TritonX100 (Roth, Karlsruhe, Germany) in PBS, followed by washing in 0.1% Tween20 (Roth, Karlsruhe, Germany) in PBS buffer and further incubation with fluorochrome conjugated secondary antibodies (1:200, Dianova, Hamburg, Germany) and counterstaining of DNA with DAPI.

For examination of cell cycle progression and DNA damage, 1000 cells were counted in cardiomyocyte-rich areas per dose and independent experiment using a Zeiss Observer microscope for epifluorescence.

2.4. Apoptosis

For determination of apoptosis, the TUNEL (TdT-mediated dUTP nick end labeling) assay was performed according to manufacturer's manual (Promega, Mannheim, Germany), cell nuclei were counterstained with DAPI. Additionally, caspase 3 activity was

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