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BIOLOGY CONTRIBUTION

MEMBRANE-DEPENDENT BYSTANDER EFFECT CONTRIBUTES TO AMPLIFICATION OF THE RESPONSE TO ALPHA-PARTICLE IRRADIATION IN TARGETED AND NONTARGETED CELLS

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Purpose: **Free radicals are believed to play an active role in the bystander response. This study investigated their origin as well as their temporal and spatial impacts in the bystander effect.**
Methods and Materials: **We employed a precise alpha-particle microbeam to target a small fraction of subconfluent osteoblastic cells (MC3T3-E1). γH2AX-53BP1 foci, oxidative metabolism changes, and micronuclei induction in targeted and bystander cells were assessed.**
Results: **Cellular membranes and mitochondria were identified as two distinct reactive oxygen species producers. The global oxidative stress observed after irradiation was significantly attenuated after cells were treated with filipin, evidence for the primal role of membrane in the bystander effect. To determine the membrane's impact at a cellular level, micronuclei yield was measured when various fractions of the cell population were individually targeted while the dose per cell remained constant. Induction of micronuclei increased in bystander cells as well as in targeted cells and was attenuated by filipin treatment, demonstrating a role for bystander signals between irradiated cells in an autocrine/paracrine manner.**
Conclusions: **A complex interaction of direct irradiation and bystander signals leads to a membrane-dependent amplification of cell responses that could influence therapeutic outcomes in tissues exposed to low doses or to environmental exposure.** © **2009 Elsevier Inc.**

Microbeam irradiation, Reactive oxygen species, Ionizing radiation, Alpha particles, Bystander effect.

INTRODUCTION

Experiments based on medium transfer from irradiated cultures and microbeam studies have revealed that traversal of the nucleus by ionizing particles is not a necessary prerequisite to elicit a radiation response. Indeed, cells growing in medium from radiation-exposed cultures, as well as nontargeted cells in the neighborhood of irradiated cells, exhibit responses similar to those of irradiated cells. The term radiation-induced bystander effect (RIBE) is now generally used to describe these processes and includes as endpoints changes in gene expression and cell growth, increased micronuclei formation, enhanced cell death, induced sister chromatid exchanges, exacerbated mutagenesis, and triggered genomic instability (1, 2).

The type and extent of RIBE were shown to be influenced by the techniques used and several of the applied parameters. Mainly, RIBE depends on gap-junctional intercellular communication and the transfer of secreted soluble factors through the medium. Moreover, RIBE depends on emitter and receiver cell types (3), and cellular consequences measured in bystander cells could vary depending on cell type and irradiation procedure (4). Medium transfer was one of the first approaches used in RIBE studies. The development of microbeam approaches together with cell imaging has proved to be particularly fruitful for characterizing the simultaneous transmission of bystander signals through the gap junctions of spatiotemporal well-defined neighboring cells (4, 5). These conditions *in vitro* may well mimic the complex intercellular relations that occur in tissues.

A growing amount of evidence indicates radiation-induced effects in nontargeted cells; pioneering works, notably those of Lyng *et al.* (6, 7), began to clarify some of the mechanisms involved. Besides inducing double-strand breaks (DSBs), ionizing radiation also induces oxidative metabolism, predominantly involving reactive oxygen species (ROS) or

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reactive nitrogen species (RNS). It triggers numerous signaling events or pathways originating in the nucleus, cytoplasm, plasma membranes, and mitochondria of targeted cells (8, 9). The impact of the induction of metabolic ROS/RNS in bystander signaling and on cell culture is not well characterized. Moreover, in most studies, involvement of oxidative metabolism was based on bystander response inhibition when scavengers such as dimethyl sulfoxide or superoxide dismutase (SOD) was used (10) but rarely by performing direct observations. To date, ROS producers are supposed be activated by transforming growth factor-β (TGF-β) and gap junctions but also by secreted ROS/RNS, lipid rafts, and calcium fluxes (4, 10, 11).

In the present study, we evaluated the impact of RIBE in cell responses to alpha-particle irradiation of subconfluent cultures of normal osteoblastic cells (MC3T3-E1). We employed an accelerator-based microbeam which selectively targets preset fractions of cells with 10 alpha particles per nuclei to characterize cell response and study how the response varies depending on cell environment. We first established the time course of biological consequences related to global and mitochondrial oxidative stresses, as well as DSB foci, in a time period of up to 24 hours, both in the targeted cells and the bystander cells in order to identify ROS producers involved in the bystander response. We then focused on the contribution of membrane signaling by using filipin treatment in the induction of ROS and DSBs and their involvement in inducing micronuclei at 24 hours after irradiation in bystander and targeted cells.

METHODS AND MATERIALS

Cell culture and treatments

MC3T3-E1 cells were from the American Type Culture Collection. Cells were grown in alpha-modified Eagle's minimal essential medium (catalog no. M8042; Sigma Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 20 μg/ml kanamycin-sulfate (Sigma Aldrich). Cells were maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air and subcultured every 3 days by dissociating with 0.25% (wt/vol) trypsin-EDTA. Asynchronous cells destined for alpha-particle irradiation were cultured in specially constructed dishes containing in the center a 2.25-mm^2 surface and a 200-nm-thick Si_3N_4 window precoated with polylysine to facilitate cell attachment. Cells were seeded at a density of about 1.50×10^4 cells/cm^2. In some experiments, cells were treated with 0.5 μg/mL filipin (Sigma) for 15 min before irradiation.

Microbeam irradiation

The layout and methods for irradiation using the microbeam have been described previously (12). The microbeam irradiation device was developed by taking into account essential conditions for cell culture (temperature at 37°C and gases and humidity control).

Briefly, approximately 350 MC3T3-E1 cells were seeded overnight, so that cell cultures were at a confluence of 80% at the time of irradiation. For nuclear irradiation, cells were stained for 30 min with 1 μM of Hoechst 33342 solution and then washed with medium. The location of individual nuclei in each cell was determined and stored by optical imaging of the fluorescent staining pattern between 330 and 385 nm. Cells were viewed with a sensitive integrating charge-coupled device camera allowing low-intensity illumination. An image analysis system using Image Pro Plus software with a computer-controlled stage was used to position cells with an accuracy of ± 1 μm over the collimated alpha-particle beam (typical hit precision, 10 μm).

Irradiation was performed with 10% of the cell population, except for a micronuclei assay, where 10% to 100% of cells were irradiated using 3-MeV alpha particles with a fluency tuned to 10 alpha particles/nucleus. Considering observations by Gault *et al*. (13) regarding potential conjugated effects when stained cells are exposed to UV, we systematically performed a sham irradiation for each irradiated culture, using a control culture stained and exposed to UV under equal conditions (one exposure). We defined targeted cells as cells receiving 10 alpha particles and bystander cells as any cell located within a radius of 150 μm from targeted cells. Using the cells positions that were stored, we performed a cell-by-cell analysis directly on the Si_3N_4 culture window.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 10 min. Cells were blocked in 3% milk in a phosphate-buffered saline (PBS) (Sigma Chemical Co.) solution for 20 min and incubated for 90 min at room temperature with the following primary antibodies: mouse monoclonal γH2AX (Ser 139), 1:500 (Upstate Biotechnology); and rabbit polyclonal 53BP1 (H-300), 1:300 (Santa Cruz Biotechnology). Secondary antibodies labeled with fluorescein isothiocyanate (Sigma) or Texas Red (Santa Cruz Biotechnology) were added at 1:1,000, and slides were incubated at room temperature for 1 hour. Slides were mounted with Glycergel mounting medium, aqueous (Dako). Images were acquired at room temperature with a Zeiss Imager M1 microscope equipped with a Zeiss Plan-Neofluar 40× oil objective lens and a Zeiss Axiocam camera under the control of Axiovision version 4.2 software. Image processing with ImageJ software was applied to whole images only. Images used for comparison between different treatments were acquired with the same instrument settings and exposure times and were processed similarly. Merges of 53BP1 and γH2AX foci were considered DSB repair foci and were numbered in each nucleus. Then, the mean number of foci per nucleus was calculated for each cell population.

Cell loading with fluorescent probes

A combination of three fluorescent probes (Molecular Probes, Inc.) was used to assess oxidative stress. At appropriate time points, cells were loaded with 7-amino-4-chloromethylcoumarin (CMAC), 5-(and-6)-chloromethyl-2′7′-dichlorodihydro-fluorescein-diacetate-acetyl-ester (CM-H2DCFH-DA), or dihydrorhodamine 123 (DHR123). These dyes freely diffuse through the membrane of living cells. Once inside the cells, the dyes are cleaved by cellular esterases, and excited probes emit fluorescence when oxidized by ROS or reduced glutathione (GSH).

CMAC was employed to determine variations in reduced GSH levels in cells. The reagent was prepared as a 2 mM stock solution in dimethyl sulfoxide and used at 100 μM in warm culture medium. After loading, the marker was removed by several washes with medium, and cells were immediately fixed with glutaraldehyde for 1 hour. Cells were then stained using 10 μM propidium iodide for 10 min. Si_3N_4 windows were mounted with Glycergel mounting medium, aqueous (Dako).

We employed CM-H2DCFH-DA to measure the intracellular generation of ROS/RNS in whole, living cells. A stock solution of CM-H2DCFH-DA was prepared in dimethyl sulfoxide on a daily