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Bystander response in human lymphoblastoid TK6 cells

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Abstract

The mechanisms of the medium-mediated bystander response induced by γ-rays in non-irradiated TK6 cells were investigated. Cell cultures were irradiated and the culture medium discarded immediately after irradiation and replaced with a fresh one. In cells incubated with conditioned medium from irradiated cells (CM), a significant decrease in cell viability and cloning efficiency was observed, together with a significant increase in apoptosis, also in directly irradiated cells. To examine whether bystander apoptosis involved the extrinsic pathway, an inhibitor of caspase-8 was added to CM cultures, which significantly decreased apoptosis to control levels. The addition to CM of ROS scavengers, Cu–Zn superoxide dismutase and *N*-acetylcysteine did not affect the induction of apoptosis. To assess whether CM treatment activates a DNA damage response, also the formation of γ-H2AX foci, as markers of double-strand breaks and their colocalisation with 53-binding protein 1 (53BP1) and the protein mutated in the Nijmegen breakage syndrome 1 (NBS1) was analysed. In cultures treated for 2 h with CM, 9–11% of cells showed γ-H2AX foci, which partially or totally lacked colocalisation with 53BP1 and NBS1 foci. About 85% of irradiated cells were positive for γ-H2AX foci, which colocalised with 53BP1 and NBS1 proteins. At 24 h from irradiation, very few irradiated cells retained foci, fitting DNA repair kinetics. The number of foci-positive bystander cells also decreased to background values 24 h after CM incubation. Our results suggest that irradiated TK6 cells release into the medium some soluble factors, not ROS, which are responsible for the cytotoxic effects induced in bystander cells. In our experimental system, the role of ROS appeared to be of minor importance in inducing cell mortality, but probably critical in activating the DNA damage response in the responsive fraction of bystander cells.

Keywords: Bystander effect; γ-Rays; Apoptosis; DNA damage

1. Introduction

Experimental data collected over the last 10–15 years have shown that ionising radiation can induce biological responses even in non-irradiated cells, so-called non-targeted responses, which include the adaptative response, bystander effects, and genomic instability [1–3]. The bystander effect (BE) is the biological response of non-irradiated cells induced by contact with irradiated cells. The contact with bystander factors may occur by direct cell–cell interaction or be mediated by the fluid surrounding the cells. It has been reported that the BE causes cell death, cell cycle arrest, apoptosis, and changes in gene expression, and increases micronucleus induction, chromosomal aberrations, mutation frequency, and DNA damage in cells neighbouring hit cells [3–14]. Evidence is accumulating that multiple signal transduction pathways are involved in this process, including gap junction-mediated intercel-

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lular communication, oxidative metabolism, and soluble extracellular factors [15,16].

Direct evidence of the bystander effect has been provided by *in vitro* studies, in which the conditioned medium from irradiated cells was transferred to non-irradiated cultures, or cell populations were irradiated and immediately mixed with non-irradiated ones [5,7,17–19]. Advances in the understanding of the BE mechanism have been gained through the microbeam system, which irradiates individual cells or subcellular targets with a defined number of charged particles. The effects can be recorded separately, in both cells targeted with ionising radiation and neighbouring non-irradiated cells [20–24].

Conclusive evidence has been obtained for the existence of radiation-induced bystander effects, but the mechanisms that trigger and regulate these processes are still largely unknown. Reactive oxygen species (ROS) originating in irradiated cells can induce oxidative stress in non-irradiated cells, as reported by many investigators [10,15,25]; in addition to ROS, irradiated cells may release soluble factors which are toxic for bystander cells into the medium [16]. Differences in bystander responses among various cell types are thought to be related to DNA repair capacity, suggesting the fundamental role of DNA damage in inducing BE [16,11,26].

DNA double-strand breaks (DSBs) are frequently used as markers of DNA damage, since they can reveal the phosphorylated form of the histone variant H2AX (γ-H2AX), which forms foci over large chromatin domains surrounding DSBs. The phosphorylation of H2AX at serine 139 by members of the phosphatidylinositol-3-OH kinase (PI(3)K)-like family (ATM, DNA-PK, and ATR) is one of the earliest steps in the cellular response to DNA DSBs [27,28]. Several proteins of DNA repair and cell cycle checkpoints are recruited at the γ-H2AX foci, forming ionising radiation-induced foci (IRIF), which play a critical role in the retention of repair factors at DSB sites, as well as amplification of the damage-induced checkpoint signal [29–31]. The kinetics of the formation and disappearance of γ-H2AX foci closely parallel the rate of DSB repair [32].

The aim of our work was to study the mechanisms of bystander responses induced by conditioned medium from γ-irradiated cells to non-irradiated TK6 cells. To limit the involvement of ROS generated during irradiation in inducing the bystander response, the medium was discarded immediately after irradiation and replaced with a fresh one. The conditioned medium from irradiated cells (CM) and the medium irradiated without cells (IM) were used to treat non-irradiated cultures, in which cytotoxicity and the induction of apoptosis were assessed. To evaluate the role of ROS in inducing bystander apoptosis, ROS scavengers, Cu–Zn superoxide dismutase (SOD) and *N*-acetylcysteine (NAC) were added during incubation with CM or IM. The inhibitor of caspase-8, Z-IETD-fmk, was also added during incubation, to verify whether the extrinsic pathway was involved in bystander apoptosis.

To assess whether CM treatment could activate DNA damage in bystander cells, we analysed the formation of γ-H2AX foci, as markers of DSBs, and their colocalisation with 53-binding protein 1 (53BP1) and NBS1-p343, the protein mutated in the Nijmegen breakage syndrome 1 phosphorylated at Ser343, both of which are involved in the early steps of DNA repair and checkpoint activation. 53BP1 binds to γ-H2AX and rapidly colocalises at IRIF, where it coordinates both DNA repair and G2/M arrest [33,34]. The number as well as the formation and disappearance of 53BP1 foci, almost completely matched that of γ-H2AX in cells exposed to γ-irradiation and other DNA-damaging agents [35]. The NBS1 protein is a component of the trimeric complex MRE11-RAD50-NBS1 (MRN) that, in mammalian cells, functions at an early stage of homologous recombination, as well as in checkpoint activation mediated by the protein kinase ATM (Ataxia-Telangiectasia-mutated) [36].

Our data show that irradiated TK6 cells release into the culture medium soluble molecules, not only ROS, which maintain cell mortality high in bystander cells for at least 48 h. DNA lesions detected in bystander cells are transient, and are probably generated by the short-lived ROS released by irradiated cells.

2. Materials and methods

2.1. Cell cultures and irradiation

Human TK6 lymphoblastoid cells (American Type Culture Collection, CRL 8015, p53 wild type), derived from the male donor WIL-2 [37], were cultured at 37 °C in RPMI 1640 medium containing GlutaMAX I (Gibco, Invitrogen) and supplemented with 10% heat-inactivated foetal bovine serum (FBS, Seromed) in a humidified incubator in an atmosphere of 5% CO_2 in air. Exponentially growing asynchronous cell populations were irradiated with 0.5–1 Gy with γ-rays in RPMI medium at 1×10^6 cells/ml. Irradiations were carried out with the ^{60}Co "gamma beam" of CNR-FRAE at the INFN-Laboratori Nazionali di Legnaro (Padova, Italy) and at the Department of Oncological and Surgical Sciences of the University of Padova from a ^{137}Cs source (dose-rate: 1 Gy/min).

2.2. Media transfer

Immediately after irradiation at 0.5 Gy and 1 Gy, cells were centrifuged, resuspended at 0.5×10^6/ml in fresh medium, and