Mutation Research 789 (2016) 39–47

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Vincristine-induced bystander effect in human lymphocytes

Serena Testi[a], Alessia Azzarà[a], Caterina Giovannini[a], Sara Lombardi[a], Simona Piaggi[c], Maria Sole Facioni[a], Roberto Scarpato[a,b,*]

[a] Unità di Genetica, Dipartimento di Biologia, Pisa University, Via Derna 1, 56126 Pisa, Italy
[b] Research Center of Nutraceuticals and Food for Health, University of Pisa, Pisa, Italy
[c] Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, Pisa University, Via Savi 10, 56126 Pisa, Italy

ARTICLE INFO

Article history:
Received 28 September 2015
Received in revised form 11 February 2016
Accepted 24 March 2016
Available online 25 March 2016

Keywords:
Bystander effect
Micronuclei
Spindle poisons
Human lymphocytes
ROS
Cytokines

ABSTRACT

Bystander effect is a known radiobiological effect, widely described using ionizing radiations and which, more recently, has also been related to chemical mutagens. In this study, we aimed to assess whether or not a bystander response can be induced in cultured human peripheral lymphocytes by vincristine, a chemotherapeutic mutagen acting as spindle poison, and by mitomycin-C, an alkylating agent already known to induce this response in human lymphoblastoid cells. Designing a modified *ad hoc* protocol for the cytokinesis blocked micronucleus (MN) assay, we detected the presence of a dose-dependent bystander response in untreated cultures receiving the conditioned medium (CM) from mitomycin-C (MMC) or vincristine (VCR) treated cultures. In the case of MMC, MN frequencies, expressed as micronucleated binucleates, were: 13.5 ± 1.41 at 6 μM, 22 ± 2.12 at 12 μM or 28.25 ± 5.13 at 15 μM vs. a control value of 4.75 ± 1.59. MN levels for VCR, expressed as micronucleated mononucleates were: 2.75 ± 0.88 at 0.0 μM, 27.25 ± 2.30 at 0.4 μM, 46.25 ± 1.94 at 0.8 μM, 98.25 ± 7.25 at 1.6 μM. To verify that no mutagen residual was transferred to recipient cultures together with the CM, we evaluated MN levels in cultures receiving the medium immediately after three washings following the chemical treatment (unconditioned medium). We further confirmed these results using a cell-mixing approach where untreated lymphocytes were co-cultured with donor cells treated with an effect-inducing dose of MMC or VCR. A distinct production pattern of both reactive oxygen species and soluble mediator proteins by treated cells may account for the differences observed in the manifestation of the bystander effect induced by VCR. In fact, we observed an increased level of ROS, IL-32 and TGF-β in the CM from VCR treated cultures, not present in MMC treated cultures.

1. Introduction

Over the last twenty years many authors have shown that unexposed cells in irradiated cultures can experience significant biochemical and phenotypic changes that are often similar to those observed in targeted cells; this event is known as bystander effect [1–5]. According to the current model, several factors such as reactive oxygen or nitrogen species (ROS or RNS) and cytokines are produced by targeted cells in response to the radiation-induced damage [6,7]. These signals can reach neighbouring cells via gap-junctions or via the extracellular medium, and are able to induce a number of different effects in the unexposed cells such as sister chromatid exchanges [8,9], micronuclei [10], apoptosis [11] or changes in patterns of gene or miRNA expression [12,13]. Several studies have also demonstrated the existence of the bystander effect *in vivo*, highlighting the relevance of this response in therapies based on the irradiation of neoplastic tissues [14–18], thus providing important hints for the optimization of radiotherapy. As cancer therapies are often based on the use of chemotherapeutic drugs, it would also be important to understand if these molecules can also induce a bystander effect. In this regard, melanoma cultures from mice treated with *N*-chloroethyl-*N*-nitrosourea induced growth inhibition, cytoskeletal and metabolic alterations in bystander cells [19]. In addition, mitomycin-C and phleomycin were able to increase micronuclei frequency in bystander cells and to provoke the activation of MAPK pathways [20,21]. Other studies have shown that the treatment with actinomycin-D conditioned medium can induce the activation of the intrinsic apoptotic pathways [22]. More recently, the exposure of several cell types to different concentrations of bleomycin and neocarzinostatin, produced the bystander effect in cells not

* Corresponding author at: Unità di Genetica, Dipartimento di Biologia, University of Pisa, Via Derna 1, 56126 Pisa, Italy.
E-mail address: roberto.scarpato@unipi.it (R. Scarpato).

http://dx.doi.org/10.1016/j.mrfmmm.2016.03.001
0027-5107/

receiving the mutagens, increasing the rate of micronuclei [23]. Bearing this background in mind, we aimed to verify whether or not the bystander effect was induced in cultures of peripheral blood lymphocytes by using, as a reference or test chemical, the bi-functional alkylating agent mitomycin-C (MMC), already known to induce the bystander response, or the spindle poison vincristine (VCR), respectively. VCR prevents the polymerisation and depolymerisation of microtubules, hence inhibiting their dynamic instability, which is essential for the formation of the mitotic spindle [24].

After analysing the induction kinetics of the effect in the reference mutagen, 1) we attempted to detect the presence of a bystander response, expressed as micronuclei (MN) formation, transferring medium from treated to untreated cultures (principal experiments), 2) we confirmed the results using a cell-mixing approach, 3) we analysed the type of MN induced in bystander cultures, and 4) we assessed the involvement of ROS and soluble mediator proteins (IL-32 and TGF-β), which may be involved in the manifestation of the observed effect. To do this, we performed the entire study under standardized experimental conditions, obtained by designing a modified *ad hoc* protocol for the cytokinesis blocked micronucleus assay (CBMN), a well-characterized assay to detect genotoxic and cytotoxic damage [25,26].

2. Materials and methods

2.1. Cell cultures, treatment and harvesting

Heparinised whole blood samples were obtained by venipuncture from four healthy 23- to 26-year-old donors (3 males and 1 female) previously proved to give a comparable response to mutagen treatment. The study was performed according to the Pisa University Ethical Committee.

Two experiments, consisting of a series of two independent cultures per experimental point, were performed for each mutagen treatment. One male and the female were used in all the cell-mixing experiments, the other two males were used in all the conditioned medium experiments. Each culture tube was set up with 300 μl of whole blood and 4.7 ml of RPMI-1640 medium (Life Technologies, Milan, Italy) supplemented with 20% foetal bovine serum (Life Technologies, Milan, Italy), 1% antibiotic/antimitotic (Life Technologies, Milan, Italy) and 1.5% phytohaemagglutinin (Life Technologies, Milan, Italy) and incubated at 37 °C for a total time of 72 h. Independently of the specific experimental approach applied, cultures were treated after 24 h from the start of culturing with different doses of mitomycin-C (6, 12 and 15 μM, final concentration) (MMC; Sigma-Aldrich, Milan, Italy) and vincristine (0.2, 0.4, 0.8 and 1.6 μM, final concentration) (VCR; Sigma-Aldrich, Milan, Italy) for 1 and 2 h, respectively. All mutagens were dissolved in sterile H_2O and control cultures not treated with mutagens were also set up. To block cell cytodieresis, cytochalasin B (Cyt B; Sigma-Aldrich, Milan, Italy, 6 μg/ml final concentration) was added at 44 h, and all cultures were harvested at 72 h (end of cell culturing). Lymphocytes were harvested by 4-min centrifugation at 2400 rpm, and the remaining cell pellet was treated with 5 ml of 0.075 mM KC1 for a few min to lyse erythrocytes, prefixed in methanol/acetic acid (3:5), re-centrifuged, fixed in 100% methanol for at least 30 min, washed twice in methanol/acetic acid (9:1), and dropped onto clean glass slides. Harvesting procedure was performed at room temperature.

2.2. Conditioned medium transfer

As shown in Fig. 1, for each mutagen we set up two groups of cultures, *donor cultures* (DC) and *recipient cultures* (RC). DC were cultures in which cells come in direct contact with the chosen mutagen. To remove the mutagen at the end of the treatment, DC underwent three rounds of washes, each consisting in a 4-min centrifugation, removal of the supernatant and re-suspension of the cell pellet in 10 ml of fresh medium. Then, according to the standard procedure described elsewhere [27], each DC tube was passed through a 0.20 μm sterile filter (Sarstedt, Verona, Italy) to ensure that no cell factors/debris remained in the media. The filtered medium harvested from these cultures (called *conditioned medium,* CM) was transferred to the corresponding RC at different established times ($T_{transf} > 0$ h) measured after the end of the three washes. RC were then analysed for the induction of a bystander response. To avoid the possibility that residuals of chemicals might be responsible for the effect observed in RC, we also set up a parallel series of cultures called respectively *washing donor cultures* (WDC) and *washing recipient cultures* (WRC) that reproduced the same experimental procedures as DC and RC with the difference that WRC received the medium from WDC immediately after the washes ($T_{transf} = 0$ h) in order to prevent bystander signalling molecules being released in the medium.

2.3. Temporal kinetic and bystander effect induction analysis by conditioned medium transfer

For the temporal kinetic analysis, we treated only DC with the reference mutagen MMC. At the end of treatment, DC were washed

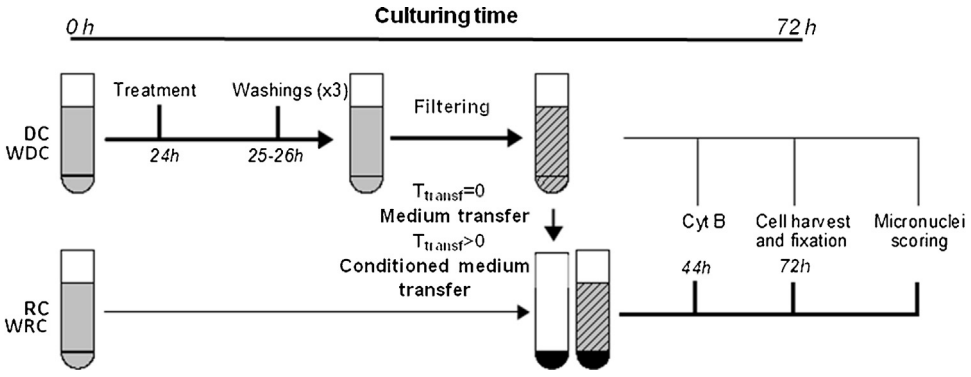

Fig. 1. Schematic representation of the protocol adopted in medium transfer experiments with the mutagens mitomycin c (MMC) and vincristine (VCR). The *donor cultures* (DC) and *recipient cultures* (RC) were incubated at 37 °C (0 h) up to 72 h. At 24 h DC were treated for 1 h (MMC) and 2 h (VCR). After the treatment, the mutagen was washed away and the *conditioned medium* (CM) was transferred to the corresponding RC at $T_{transf} > 0$ h (1.0, 1.5, 2.0 and 2.5 for the temporal kinetic analysis, and 1.5 h for the bystander experiments). RC were then analysed for the induction of a bystander response. Cyt B was added to all cultures at 44 h. After cell harvesting (72 h), slides were set up for micronuclei analysis. A parallel series of cultures called respectively *washing donor cultures* (WDC) and *washing recipient cultures* (WRC) were also set up, which underwent the same procedures described for DC and RC with the difference that WRC received the medium from WDC immediately after the washings ($T_{transf} = 0$ h).