Mutation Research xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

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

Human brain glioblastoma cells do not induce but do respond to the bleomycin-induced bystander response from lung adenocarcinoma cells

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ARTICLE INFO

Article history:
Received 3 November 2012
Received in revised form 16 July 2013
Accepted 20 July 2013
Available online xxx

Keywords:
Bleomycin
Glioblastoma
Bystander response
Micronucleus
H2AX
Cell cycle kinetics

ABSTRACT

To determine whether the bleomycin (BLM)-induced bystander response occurs in human brain glioblastoma (BMG-1) cells, the BMG-1 cells were exposed to two different concentrations of BLM. The co-culture methodology was adopted to study the in vitro bystander effects. DNA damage was measured using the micronucleus (MN) and γ-H2AX assays. Cytotoxicity was measured using the trypan blue assay. Cell cycle kinetics was analyzed using flow cytometry. The overall results did not show any significant increase in either genotoxicity or cytotoxicity or a delay in the cell cycle kinetics in BMG-1 bystander cells co-cultured with BLM-exposed cells, suggesting that BLM did not induce a bystander response in the BMG-1 cells. Furthermore, the MN results of the BLM-exposed BMG-1 cells co-cultured with unexposed bystander human lung adenocarcinoma (A549 and NCI-H460) cells and vice versa suggested that the BMG-1 cells do not secrete bystander signals but do respond to those signals. Analyzing the underlying mechanism and pathways involved in preventing the cells from secreting bystander signals will provide new insights that can be applied to inhibit these mechanisms in other cell types, thereby preventing and controlling the bystander response and genomic instability and increasing the therapeutic gain in chemotherapy.

1. Introduction

Second malignancies occurring after primary cancer treatment have become a major concern during the past decade. The radiation-induced bystander effect (the expression of a radiation signature in unexposed cells that have received signals from the exposed cells) has been proposed as a possible mechanism for the incidence of second malignancies. Similarly, certain chemotherapeutic agents, such as phleomycin [1], actinomycin D [2], mitomycin C [3], chloroethylnitrosourea [4], paclitaxel [5] and FOLFOX [6], also induced the bystander response. Recent progress in the phenomena of the chemotherapeutic drug-/radiation-induced bystander effect has shown its association with genomic instability, which is ultimately accepted as the initiating event for carcinogenesis [7,8].

Bleomycin (BLM) is a chemotherapeutic agent used to treat squamous cell carcinomas, melanoma, sarcoma, Hodgkin's and non-Hodgkin's lymphomas and testicular cancer [9] alone or in combination with other drugs [10,11]. BLM has been shown to induce a 7% risk of a second malignancy [12]. With respect to the chemotherapy, we observed bystander effects in normal human lung fibroblasts (WI-38), peripheral blood lymphocytes (PBL), human bone marrow mesenchymal stem cells (hBMSC) and lung adenocarcinoma cells (A-549, NCI-H460) exposed to chemotherapeutic agents, such as BLM and neocarzinostatin (NCS) [13] and also in cells grown in three-dimensional architecture to mimic the in vivo tumor microenvironment [14]. We further analyzed the involvement of reactive oxygen and nitrogen species in mediating the BLM-induced bystander response in human acute lymphocytic leukemia cells (CCRF-CEM) and human promyelocytic leukemia cells (HL-60) [15].

Although the bystander response phenomenon has been observed widely in many cellular models exposed to a variety of radiation types, few studies have failed to show these responses and it became evident that not all cell types produce bystander signals and/or do not respond to these signals [16,17]. Thus, the available literature implied that the radiation-induced bystander effects are highly variable, dependent on the individual donors or cell lines tested or dependent upon the adopted methodology or linear energy transfer (LET) [18–20]. Similar to the radiation-induced bystander response, to study the cell line dependency, if any, for the chemotherapy drug-induced bystander effect, we analyzed the BLM-induced bystander response in human glioblastoma (BMG-1) cells.

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http://dx.doi.org/10.1016/j.mrgentox.2013.07.005

2. Materials and methods

2.1. Cell culture and maintenance

BMG-1 cells were received as a gift from Dr. B.S. Dwarakanath, INMAS, DRDO, India. The A-549 and NCI-H460 cells were obtained from the National Centre for Cell Sciences (Pune, Maharashtra, India). The SiHa cells were a generous gift from Mr. Sambantham, Department of Molecular Biology, University of Madras, India. The BMG-1 (p18–p20), A549 (p49–p55) and SiHa (p16–p25) cells were grown in plastic tissue culture flasks using Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA). The NCI-H460 (p78-p90) cells were grown in Roswell Park Memorial Institute-1640 (RPMI-1640) (GIBCO). All the cell lines were supplemented with 10% fetal bovine serum (FBS) (GIBCO) and antibiotics (penicillin 50 IU/ml, streptomycin 35 μg/ml and gentamycin 2.5 μg/ml; GIBCO) in a humidified incubator at 37 °C with 5% CO_2.

2.2. PBL

Approximately 10 ml of human PBL was collected in a heparinized sterile container from healthy male volunteers aged 25 years with informed consent and clearance from the Institutional Ethics committee. For the experiments, the cells were grown in RPMI-1640 medium supplemented with 20% FBS and antibiotics (penicillin 50 IU/ml, streptomycin 35 μg/ml and gentamycin 2.5 μg/ml) in a humidified 37 °C incubator with 5% CO_2.

2.3. BLM or NCS exposure and co-culture of cells

The co-culture methodology described by Geraschenko and Howell [21] was adopted in the present study. Except for PBL, the cells were grown in 2 ml of growth medium in T_{25} flasks, trypsinized and seeded ($\sim 1 \times 10^5$) into commercially available transwell culture inserts (Thincert™, Greiner Bio One, Germany) with a permeable membrane (pore size 0.4 μm). The cells were incubated at 37 °C for 24 h. At the same time, an equal number of cells were plated into six-well plates. After 24 h of growth, the cells seeded onto the transwell culture inserts alone were exposed to BLM (Dabur, Solan, India) or NCS (Sigma, St. Louis, MO, USA) at concentrations of 0, 40 and 80 μg/ml or 0, 2 and 4 μg/ml, respectively, for 3 h. For PBL, approximately 1 ml of blood in 2 ml of growth medium was added to the transwell culture inserts, exposed to the same concentrations of BLM and co-cultured in a similar manner. Following exposure, the drug-treated cells in the transwell culture inserts (directly exposed) were washed 3 times with their respective media without serum and were placed into the wells of the six-well plate containing untreated (bystander) cells and co-cultured for 24 h.

2.4. Quantification of bystander effect using the micronucleus assay (MN)

24 h after the co-culture, the cells (BMG-1, A-549, NCI-H460 and SiHa) were detached using trypsin–EDTA (GIBCO), collected after centrifuging (1000 rpm/5 min) and counted using a hemocytometer. Approximately 1×10^5 cells were seeded into P-60 dishes with their respective media and were incubated at 37 °C in a 5% CO_2 incubator. To arrest the cells at the cytokinesis stage, cytochalasin-B (3 μg/ml) (Sigma, Bellefonte, PA, USA) was added to the medium. After 48 h of incubation, the cells were washed with phosphate buffer saline (PBS), fixed with ice-cold methanol and air dried. Then, the cells were stained with diamino-phenyl-indole (DAPI) (Vysis Inc., Downers Grove, IL, USA) and analyzed using a fluorescence microscope (40×) with an appropriate filter. For PBL, 3 μg/ml cytochalasin B was added at the 44th hour to arrest the cells at the cytokinesis stage. After 72 h of incubation, the cells were centrifuged (800 rpm/8 min), washed with ice cold hypotonic 0.075 M KCl solution and fixed with ice-cold Carnoys fixative (3:1). The cells were cast on clean glass slides, stained using Giemsa stain and analyzed for the presence of MN using a light microscope (40×). A total of 1000 binucleated cells for each treatment group were scored and the MN frequency was calculated.

2.5. Quantification of bystander effect using γ-H2AX foci frequency by immunofluorescence

Approximately 1×10^6 BMG-1 cells were used for the expression measurement of γ-H2AX foci. The cells were fixed in 2% paraformaldehyde and ice cold methanol and were permeabilized using 0.5% Triton X-100. Then, the cells were blocked with 1% bovine serum albumin, washed and incubated with the primary antibodies [anti-H2AX (Abcam, Cambridge, UK)] followed by incubation in the secondary antibody labeled with isothiocyanate fluorescence (Abcam) for 1 h at 4 °C in the dark. Flow cytometry was performed using a FACS Calibur (Becton Dickinson Systems) and 10,000 events were scored. The analysis was performed using Cell Quest software. As the resulting histogram was characterized as monophasic, the mean fluorescence ratio was calculated using relative FITC fluorescence intensities, as described earlier [22].

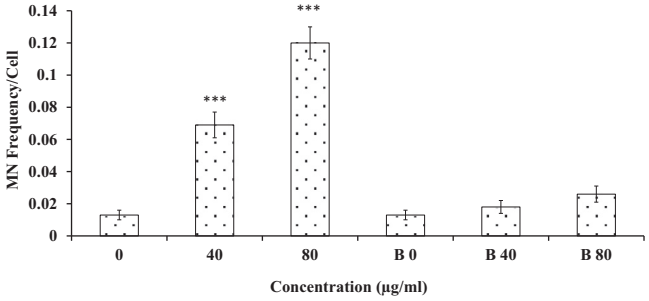

Fig. 1. Comparison of the MN frequency obtained in the BMG-1 cells grown on transwell culture inserts, exposed to 0, 40 and 80 μg/ml of BLM for 3 h and then co-cultured with their respective bystander cells (B0, B40 and B80) for 24 h. Each bar represents the mean ± SE of the frequency of micronuclei induced for three independent experiments ($N = 3$). Significance levels of $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ were indicated in the test bars compared with their respective control bars.

2.6. Viability assay

Cell viability was analyzed by trypan-blue exclusion. The BMG-1 cells were treated directly with BLM and the bystander cells were trypsinized and suspended in 1 ml of culture medium. Approximately 100 μl of the cell suspension was mixed with an equal volume of trypan blue. Then, 20 μl of the cell suspension was loaded into a hemocytometer and the stained cells were counted using inverted phase-contract microscope. The percentage of cell viability was calculated.

2.7. Cell cycle kinetics

The BMG-1 cells exposed to BLM and their bystander cells were trypsinized thereafter, washed twice with cold PBS and centrifuged. The cells were ethanol fixed and stored at 4 °C until analysis using flow cytometry. For analysis, the cells were centrifuged at 1000 rpm for 3 min and then, the supernatant was removed. Cold PBS was added and the cells were gently resuspended. The centrifugation step was repeated and the cells were gently resuspended in PBS with 100 mg/ml RNase (Qiagen, Hilden, Germany) and incubated at 37 °C for 1 h. The samples were again centrifuged and aspirated and the remaining pellet was gently resuspended in PBS with 50 mg/ml propidium iodide (Sigma). The samples were incubated on ice for 30 min. A total of 10,000 events from each sample were analyzed using a Becton Dickinson FACS Calibur Flow Cytometry System.

2.8. Statistical analysis

All the experiments were performed three times and the average of the assimilated data was taken as the final result. The paired *t*-test and a one-way analysis of variance (ANOVA) were performed to compare the differences in the MN frequencies within and between the treatment groups for each cell type individually using the software INSTAT programmer.

3. Results

3.1. BLM exposure did not induce DNA damage in the bystander BMG-1 cells

The MN assay results showed that the obtained MN frequency did not show significant differences between control and bystander control BMG-1 cells ($p > 0.05$). A dose-dependent increase in the MN frequency was observed in the BMG-1 cells directly exposed to BLM, representing a significant increase ($p < 0.001$). In contrast to directly exposed cells, the bystander BMG-1 cells co-cultured with the directly exposed cells did not show any significant ($p > 0.05$) increase in the MN frequency compared with its control. These results suggested that the BLM did not induce the bystander effect in BMG-1 cells as measured by the MN assay (Fig. 1).

3.2. Relative fluorescence intensity of the γ-H2AX foci of BMG-1 cells exposed to BLM and their bystander cells measured using flow cytometry

To further confirm the MN assay results, γ-H2AX foci formation, a measure of DNA double strand breakage to quantify early

Please cite this article in press as: S.A.S. Basheerudeen, et al., Human brain glioblastoma cells do not induce but do respond to the bleomycin-induced bystander response from lung adenocarcinoma cells, Mutat. Res.: Genet. Toxicol. Environ. Mutagen. (2013), http://dx.doi.org/10.1016/j.mrgentox.2013.07.005