

BLEOMYCIN INDUCED BYSTANDER RESPONSE IN HUMAN NORMAL LUNG FIBROBLASTS (WI38) AND ADENOCARCINOMA CELLS (NCI-H23)

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ABSTRACT:

Purpose: The effects of cells directly exposed to various chemotherapeutic drugs including bleomycin (BLM) have been extensively studied in the past decade. Of late, it has been demonstrated that an existence of phenomena of bystander effect, the expression of DNA damages in cells directly not traversed by radiation has been proved in-vitro and in-vivo studies, challenges the paradigm of radiation biology and its raises the concern on the uses of radiation for cancer therapy. Since, the cancer treatment procedures include radiotherapy, chemotherapy alone or in combination in the management of malignant diseases, occurrence of bystander effects after chemotherapy might further raises concern on its use in combinational therapy. Hence, it was indented to study the bystander response of a chemotherapeutic drug, the BLM, as such studies are meager.

Material and methods: Confluent density contact inhibited normal lung fibroblasts and (WI-38) and lung adenocarcinoma cells (NCI-H23) were directly exposed to various concentrations of bleomycin and co-cultured with

cells (bystander cells) non-exposed to drug for 24 hours. Then the amount of DNA damage was studied in both cells directly exposed to drug and bystander cells using cytokinesis blocked micronucleus assay.

Results: The results showed that exposure of bleomycin induced a concentration responsiveness and significant ($P > 0.0001$) increase in DNA damage in directly treated and bystander populations of only in normal lung fibroblasts and not in adenocarcinoma cells. Further the magnitude of bystander effect is ~5 fold in co-cultured WI-38 cells 2 fold in NCI-H23 cells. The implications of the obtained results were discussed.

Discussion: Similar to radiation BLM also, induced bystander response in WI-38 and NCI-H23 cells. Understanding the mechanism of BLM-induced bystander responses may therefore highlight potential new therapeutic approaches to reduce the chance of developing therapy induced second malignancy.

Key Words: Bleomycin, Bystander response, Micronucleus.

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INTRODUCTION:

Generally, management of malignant disease is achieved by combination therapy of genotoxic agents like chemicals and radiation, which usually requires multiple cycles of treatment⁽¹⁾. The effects of cells directly exposed to radiation⁽²⁾ and chemical agents⁽³⁾ have been extensively studied in the past decade. The overwhelming view on the mechanism of cell killing is achieved by depositing high energy directly on DNA and induces breaks or free radical mediated indirect DNA damage on cells exposed to therapeutic agents. However, the paradigm of genetic alterations being restricted to direct DNA damage after exposure to ionizing radiation has been challenged by observations in which effects of ionizing radiation arise in cells that in themselves receive no radiation exposure, termed as bystander effects^(4,5). It was also observed that bystander response leads to genomic instability⁽⁶⁾ and epigenomic instability (persistent transgenerational gene expression phenotype)⁽⁷⁾ may be due to alteration of the DNA methylation. These effects are proposed as a mechanism for the development of therapy induced second malignancy.

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Ample evidence has been generated to show the existence on radiation induced bystander effects in numerous cell types exposed to both high⁽⁸⁾ and low LET ionizing radiations⁽⁹⁾ in cell line studies⁽⁸⁾ and animal experiments. Multiple biological end points like Sister Chromatid Exchanges⁽¹⁰⁾, mutations⁽¹¹⁾, micronuclei⁽¹²⁾, chromosomal aberrations⁽¹¹⁾, clonogenic survival⁽¹³⁾, H2AX assay⁽⁸⁾ gene expression changes⁽¹⁴⁾, transformation⁽¹⁵⁾ and cell proliferation⁽¹⁶⁾ have been investigated and provide concrete evidence that biological response to ionizing radiation has a contribution from un-irradiated “bystander” cells that respond to signals emitted by irradiated cells. Molecules like free radicals⁽¹⁷⁾, nitric oxide⁽¹⁸⁾ and interleukins are being implicated as the mediators of bystander response; experimental studies show that signals can be passed by cell-to-cell communication either through inter-cellular junctions⁽¹⁹⁾ or the culture medium⁽²⁰⁾ depending on the radiation type, methodology and end point used. Studies showed that reactive oxygen species (ROS) level was remain elevated at least for 24 hours in the bystander cells which may end up with genomic instability⁽¹⁷⁾. Thus majority of studies on bystander response is limited to radiation and raises concern long term consequences among general public as well patients. However, limited studies have been reported on the existence of bystander effects to chemotherapeutic drugs, though many chemotherapy drugs are in use in combination therapy for cancer. An enhanced cytotoxic effect of combined bleomycin and radiation was reported in animal models and in normal and tumor cells *in vitro*⁽²¹⁾ exposed directly. While, the radiation induced

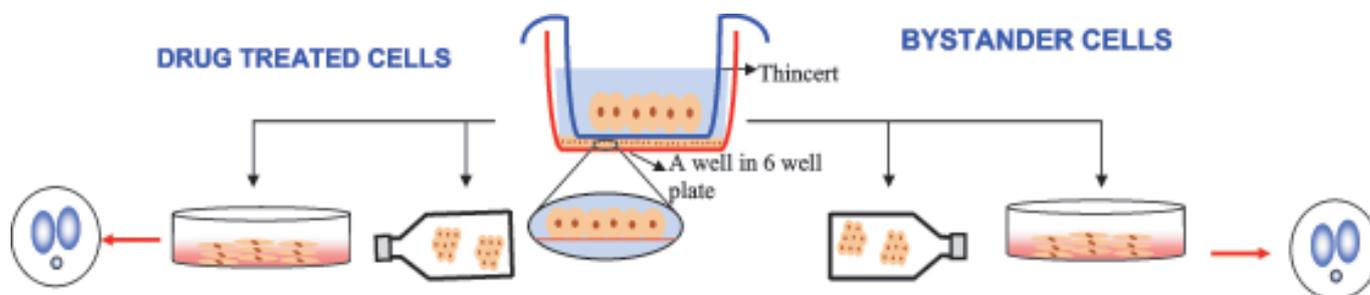
bystander effects were extensively studied in many cell systems, rare studies have been reported on the bystander effect chemotherapy drugs. Hence, in this study we made an attempt to study bystander effects induced by BLM, in human normal lung fibroblasts and lung adenocarcinoma cell lines as experimental model. The micronucleus (MN) assay was used as measure of DNA damage and bystander response in cells directly exposed and bystander population.

MATERIALS AND METHODS

Maintenance of Cells: Human normal lung fibroblasts (WI-38) cells and lung adenocarcinoma cells (NCI-H23) were obtained from national centre for cell sciences, Pune, and maintained as a monolayer culture in our laboratory. The WI-38 cells were grown in plastic tissue culture flasks using Dulbecco's modified essential medium supplemented with 10% fetal calf serum and antibiotics (Penicillin 50IU/ml, Streptomycin 35µg/ml and Gentamycin 2.5µg/ml). The NCI-H23 cells were grown in RPMI-1640 medium with the serum and antibiotics as in WI-38 culture medium.

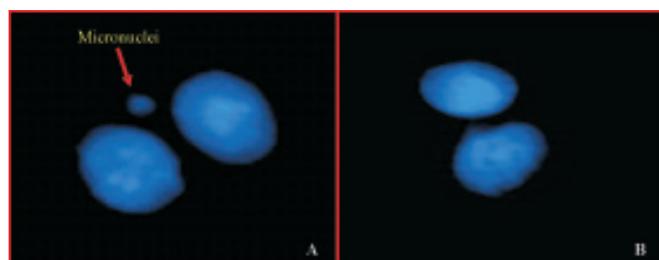
Bleomycin exposure and co-culture of cells

Figure-1 Diagrammatic illustration of co-culture methodology



Twenty four hours after co-culture, the cells were dissociated by trypsin treatment and counted using haemocytometer. About 1×10^5 cells were seeded into P-60 dishes in respective medium and incubated at 37°C in 5% CO₂. To arrest the cells at cytokinesis stage, the medium was supplemented with cytochalasin-B (Sigma) at 3 µg/ml. After 48 hours of incubation, the cells were washed with PBS and fixed in methanol. Then the cells were stained with diamino-phenyle-indole (DAPI) and coverslip was applied before the analysis. Finally, the dishes were used for the analysis of micronucleus in the binucleated cells using the fluorescence microscope (40X) with appropriate filter. At least 1000 binucleated cells were examined, and only micronuclei in binucleate cells

Figure-2 Cytokinesis arrested binucleated WI-38 cell with (A) and without (B) micronuclei.



The cells grown in T25 flasks were trypsinised and seed ($\sim 5 \times 10^5$) into thincert (Greiner Bio) placed into three wells of a 6 well plate in 2 ml of medium and incubated at 37°C for 24 hours; in parallel, equal amount of cells were plated into adjacent wells of the same 6 well plate. After 24 hours of growth, the cells seeded into thincert alone were exposed to BLM at a concentration of 40 and 80 µg/ml for 3 hours and cells in the third well was sham manipulated and used as control and maintained in 5% CO₂ at 37°C. At the end of 3 hours drug treated cells on the thincert (directly exposed cells) were washed thrice with phosphate buffered saline (PBS) and transferred to the adjacent wells and co-cultured for 24 hours with cells not treated with drug (bystander cells). To study the magnitude of bystander effect the WI-38 cells were co-cultured for 24, 48 and 72 hours and NCI-H23 cells for only 24 hours. The entire protocol is depicted diagrammatically and shown in figure-1.

Quantification of bystander effect and MN assay

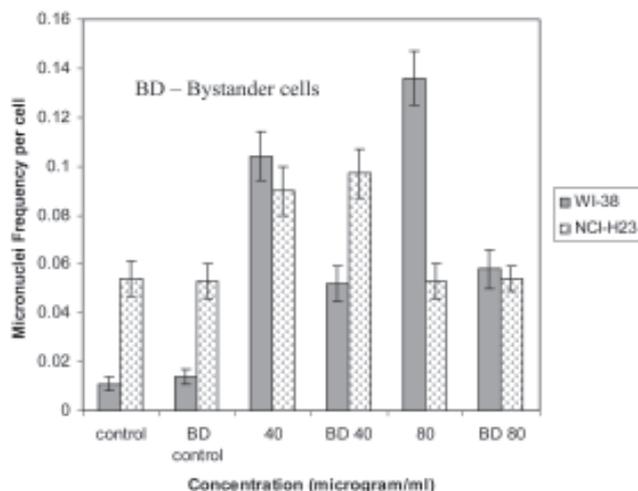
(Figure-2) were included for analysis. The frequency of micronucleus formation (r_0) was calculated as: $r_0 = a/b$, where a is the total number of micronucleated cells scored, and b is the total of binucleate cells examined. The error associated with r_0 is given by the following formula: $r_0 = ((a/b) (1 - a/b))^{1/2}$. Paired t-test (a linear regression analysis) was applied to compare the differences in the MN frequencies of different treatment groups using the INSTAT programme.

RESULTS:

MN frequency in WI-38 and NCI-H23 cells

The WI-38 and NCI-H23 cells arrested at cytokinesis stage of cell cycle are binucleate in shape and any chromosomal fragments which failed to incorporate into the daughter nucleus developed into MN as shown in figure-2 are measured to quantify the DNA damage in cells directly treated with BLM and bystander cells. The MN frequency and the standard error of mean for each data point was obtained as explained in the previous section. The MN frequency in control WI-38 cells is 0.011 ± 0.003 and in NCI-H23 cells are 0.052 ± 0.007 . Thus in the drug non-treated cells itself the baseline MN damage in lung adenocarcinoma cells is 5 fold higher than that of normal fibroblast cells. Further, co-culture of both cells, did not

Figure-3 Comparison of bystander response WI-38 and NCI-H23 cells



increase the base line DNA damage in control cells.

Bleomycin exposure induced MN in WI-38 and NCI-H23 cells

MN frequencies obtained in BLM treated WI-38 cells and NCI-H23 cells are given in tables 1 and 2 respectively. Exposure of WI-38 cells to BLM induces significantly higher ($P > 0.0001$) MN frequency that of its untreated cells and shows a concentration dependent increase. In contrast to WI-38 cells, the NCI-H23 cells did not show any concentration dependent increase in the amount of DNA damage. Among the NCI-H23 cells exposed to 40 and 80 $\mu\text{g/ml}$ of drug, cells treated with 40 μg of drug showed a

significant increase ($P > 0.001$) in MN frequency (0.99 ± 0.012) than that of drug untreated control cells; however the cells treated to 80 $\mu\text{g/ml}$, the obtained MN frequency did not show any significant difference from that of control cells (table 2).

MN frequency in bystander WI-38 cells

The frequencies of MN obtained in WI-38 cells directly exposed to drug and bystander cells (co-cultured for 24 hours) are given in table 1. While directly BLM exposed cells showed a concentration dependent increase in MN frequency, the bystander cells did not show a similar trend. It is further significant to note that the amount of DNA damage is not influenced by the co-culture duration; thus the MN frequency in the bystander cells did not show any difference while the co-culture was maintained for 48 or 72 hours when compared to 24 hours (data did not shown).

MN frequency in bystander NCI-H23 cells

MN frequencies obtained in NCI-H23 cells directly exposed to drug and in bystander cells are given in table 2. The cells co-cultured with 40 μg of drug, the MN frequency obtained is 0.108 ± 0.009 , which is almost equal to that of cells directly exposed to BLM. Similarly the cells co-cultured with 80 μg of drug, the MN frequency obtained is not statistically significant than that of directly treated cells (Table 2); however, the yield of MN frequency, is lesser than that of cells co-cultured with less concentration of drug (40 $\mu\text{g/ml}$).

Table-1 MN frequency obtained in WI-38 cells exposed to bleomycin

Concentration of BLM ($\hat{\mu}$ g/ml)		Binucleated Cells	Number of MN	MN Frequency \pm SE
0	Directly exposed cells	1000	11	0.011 ± 0.003
	Bystander cells	1000	14	0.014 ± 0.003
40	Directly exposed cells	1000	104	0.104 ± 0.01
	Bystander cells	1000	52	0.052 ± 0.007
80	Directly exposed cells	1000	136	0.136 ± 0.011
	Bystander cells	1000	58	0.058 ± 0.008

Table-2 MN frequency obtained in NCI-H23 cells exposed to bleomycin

Concentration of BLM ($\hat{\mu}$ g/ml)		Binucleated Cells	Number of MN	MN Frequency \pm SE
0	Directly exposed cells	1084	54	0.052 ± 0.007
	Bystander cells	1075	58	0.057 ± 0.007
40	Directly exposed cells	617	56	0.099 ± 0.01
	Bystander cells	1108	108	0.108 ± 0.01
80	Directly exposed cells	1072	57	0.056 ± 0.007
	Bystander cells	1195	31	0.026 ± 0.005

BLM - Bleomycin; MN - Micronucleus; SE - Standard error

Comparison of bystander response WI-38 and NCI-H23 cells

Figure-3 shows the MN frequency obtained from both cells exposed to BLM directly and co-cultured with directly exposed cells for 24 hours. In WI-38 cells the observed bystander effect in the bystander cells is five fold higher than that of bystander sham-treated control cells and not influenced by the concentration of drug to directly treated cells. In contrast, the NCI-H23 cells, showed a difference in the bystander response; that is the magnitude of response in bystander cells co-cultured with 40µg/ml treated cells showed an two fold increase than that of control bystander cells and 80µg/ml treated cells did not show any increase and remain equal to that that of control bystander cells (table-2).

DISCUSSION:

Bystander effect-like phenomena have been reported since the early 1950s, when Parsons et al⁽²²⁾ published reports on changes in sternal bone marrow following X-ray therapy of the spleen in chronic granulocytic leukemia. Further data about the interaction of cells hit and cells not hit by ionizing radiation came from Hollowell and Littlefield⁽²³⁾, in 1968, who observed chromosome damage induced by plasma obtained from patients undergoing cancer radiotherapy. Goh & Sumner⁽²⁴⁾ observed breaks in normal human chromosomes after plasma transfer from accidentally total body-irradiated patients. Radiation induced bystander effects clinical observations and experimental results concerning examples of abscopal effects have been reported by several authors, probably describing bystander effect-like phenomena, but possibly (also) inflammatory responses^(25,26). The bystander effect using high LET irradiation at very low doses was first described by Nagasawa & Little⁽¹⁰⁾. They irradiated Chinese hamster ovary (CHO) cells with very low doses (0.3–2.5 cGy) of α-particles, of which only 1% of the population was hit by an alpha particle. The authors observed an SCE frequency in the range of up to 30% of the whole population. This bystander effect occurred in vitro and saturated at 2.5 cGy. Followed which, ample evidence has been generated to show the existence on radiation induced bystander effects in numerous cell types exposed to both high⁽²⁴⁾ and low LET ionizing radiations⁽⁹⁾ in cell line studies⁽⁸⁾ and animal experiments using many end points. Existence of bystander effect is a major concern for the general public as well as the radiation regulatory agencies because of the magnified effect even though fewer cell populations are exposed.

Only few studies have focused on the ability of chemicals to induce a bystander effect; chemicals like chloroethylnitrosourea⁽²⁷⁾ mitomycin^(28,29) and phleomycin⁽²⁹⁾ have shown to induce bystander response and genomic instability in primary melanoma and lymphoblastoid cell lines respectively. It was suggested that soluble secretory factors released from the drug treated cells into the culture medium (2-6 hours), upon the transfer into non-treated cells could be possible mechanism for the observed

bystander effect. In this medium transfer experiment the action of the free radical would be limited due to its short life of 10⁻⁵seconds in the first 2-6 hours of incubation. So most of free radicals produced in the first 6 hours will be depleted without damaging the normal bystander cells which will not give the complete effect of free radicals. To overcome this, in the present study we adopted a new methodology to study the bystander response in considering the mechanism of BLM. The drug treated cells are completely separated from the bystander cells by just 1mm distance and are co-cultured in the same medium for 24 hours to study the bystander response. The magnitude of bystander effect observed is five fold higher for WI-38 cells and two fold for NCI-H23 cells. Similar reports have been shown the existence of bystander response in human normal lymphoblastoid cell line exposed chemicals and radiation⁽²⁹⁾. Their findings indicate that media conditioned by cells cultured in mitomycin-C induced a 1.5–3 fold increase in micronuclei, and that media conditioned by cells cultured in phleomycin induced a 1.5–4 fold increase, and conditioned media from irradiated cells induced a 2–8 fold increase in micronuclei compared to sham-treated controls. Both chemicals as well as radiation induced bystander effects at all the doses and concentrations evaluated. Even at the lowest levels of exposure a minimum of a 50% increase in micronuclei was observed.

The magnitude of bystander response in WI-38 cells failed to show any concentration responsiveness or duration of co-culture; this might be due to saturation effect as reported for high LET radiations⁽⁹⁾. However, the NCI-H23 cells showed the bystander response only cells co-cultured to low BLM (40 mg/ml) and the cells co-cultured with the cells exposed to 80mg/ml concentration of the drug, the MN frequency did not differ from that of control (Table-2); i.e. it did not show any bystander response. This might be attributed to cell cycle arrest or cell death, as existence of such phenomena has been reported in cells exposed to high concentration of drug and radiations. In addition, variation in the sensitivity of both cells to bleomycin, could not be ruled out as we have earlier observed BMG-1 a tumor cells of neuronal origin, could survive and express MN when exposed to 80 µg/ml concentration of the drug⁽³⁰⁾. Such a variation do drug response has been reported in-vitro^(30,31) and in patients after chemotherapy^(32,33).

The mechanisms of bystander signaling are now starting to be elucidated at the molecular level and several key molecules are known to have major roles in some systems are interleukin 6 (IL-6)⁽³⁴⁾, IL-8⁽³⁵⁾, transforming growth factor-β1 (TGFβ1)⁽³⁶⁾ and TNFα⁽³⁷⁾, ROS⁽³⁸⁾ and reactive nitrogen species^(39,18). At the molecular level, the DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) and Ataxia-Telangiectasia Mutated (ATM) are required to generate bystander signals, but not required to receive the bystander signal⁽⁹⁾ and the ATM acts in the downstream of Ataxia-Telangiectasia and Rad3-related (ATR) in the DNA damaged response signaling of bystander

cells⁽⁴⁰⁾. Cyclo-oxygenase 2 (CoX2, also known as PTGS2)-dependent signaling has been shown to be a central player in cellular inflammatory responses and also mediates bystander signaling. The activation of the MAPK pathways is crucial for the action of CoX2. Downstream signaling leads to transcription factor activation, including inducible nitric oxide (No) synthase (iNoS, also known as NoS2) activation leading to the production of reactive nitrogen species. Inhibition of these pathways in bystander cells by blocking cell-cell communication^(41,42) and by supplementing antioxidants^(42,8) leads to inactivation of bystander responses, suggesting a potential route for modulating these responses in a clinical setting⁽⁴³⁾. For example, it may be beneficial to activate bystander responses to increase tumor cell killing, or to prevent them in order to protect normal tissues from additional damage.

CONCLUSION:

The obtained results strongly supports that BLM is inducing the bystander response in WI-38 and NCI-H23 cells similar to radiation. Further, understanding the mechanism of BLM-induced bystander responses may therefore highlight potential new therapeutic approaches that invoke mechanisms related to cell-cell communication of damage-sensing signals and allow amplification of cell killing effects; if such molecules are identified, can be targeted for better management of tumor with minimal concern for late complications like therapy related second malignancy.

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