Induction of Apoptosis and Micronuclei by Bleomycin Sulfate in Different Cell Cycle Stages of Human Lymphocytes

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Abstract

Received 11/Nov/2003, Accepted 19/Dec/2003

Introduction: Bleomycin sulfate is a DNA damaging agent used in cancer chemotherapy. The effect of this drug on various cell cycle stages might be different, thus inducing different modes of death (apoptotic or mitotic death). The aim of this investigation was to study the effects of bleomycin on human peripheral blood lymphocytes at various cell cycle stages by two different end points (induction of apoptosis or micronuclei).

Material and Methods: Human peripheral blood lymphocytes were treated with various doses of bleomycin at G0, G1, and G2 phases of the cell cycle and the percentages of apoptosis (AP) and micronuclei (MN) were determined. The peripheral lymphocytes were isolated by ficoll hypaque and suspended in RPMI-1640 containing 15 % fetal calf serum. The isolated lymphocytes were stimulated by phytohemagglutinin (PHA), cultured again in RPMI-1640, harvested after 64 hrs and 96 hrs, and stained with acridine orange and ethidium bromide to determine the percentage of apoptotic cells. MN assay was done according to the standard in vitro micronucleus assay.

Results: The results showed that the percentages of apoptotic cells and MN at G2 stage were significantly higher than those of G0 and G1 stages. At higher doses, MN formation and apoptotic cells were increased; however with increasing time, the percentage of MN decreased while the percentage of apoptotic cells generally increased in all the cell cycle stages.

Conclusion: The results indicate that bleomycin is a potent inducer of both micronuclei and apoptosis. The incidence of apoptotic cells following bleomycin treatment in G0 and G1 was much higher than the incidence of micronucleated cells at the two sampling times. The percentage of AP cells following bleomycin treatment remained constant across cell cycle stages.

Key words: Bleomycin, Micronucleus, Apoptosis, G0, G1, G2- Lymphocytes.

Introduction

The cancer chemotherapy drug, bleomycin (BLM) is a potent inducer of genetic damage in a wide variety of assays. Because of its extensive use in clinical chemotherapy, its clastogenic effects have been studied extensively (1, 2). Bleomycin is a cytotoxic glycopeptide isolated from Streptomyces verticillies (3). It is an antibiotic that has antitumor activity against squamous cell carcinoma and malignant lymphomas (4). The activity of BLM is due to the effects on DNA synthesis (5, 6) and its cytotoxicity is related to DNA damage, chromosome breakage and inhibition of protein synthesis (7, 8). As well as being cytotoxic, BLM interferes with cell progression when applied to G2 cells (9, 10). It has been shown that BLM induces molecular and cytological effects similar to ionizing radiation (11, 12) such as induction of DNA strand breaks, G2 delay and chromosome aberrations (1, 13). The chemical structure of BLM consists of two domains, a DNA binding domain and an oxygen activating domain (14). BLM is a potent inducer of sister chromatid exchange (7, 15) and also MN, in vivo and in vitro (16, 17). BLM has also been shown to produce a positive response in the comet assay in human lymphocytes. BLM induces AP in many cell lines such as pancreatic carcinoma cells, mouse leukemia L1210 cells, and others (18, 19).

There is evidence that apoptosis induction by BLM may involve the formation of reactive oxygen species (ROS) (18, 20, 21). The physico-chemical information indicates that the beta- aminalaninamide- pyrimidine-beta- hydroxyhistidine region of BLM forms an iron complex to activate the oxygen (20, 14). ROS may cause mitochondrial dysfunction, which results in release of cytochrome C and activation of the caspase cascades (22). DNA binding compounds can result in chromosomal aberration that may cause either MN formation or AP (16, 23). MN which is due to acentric chromosomal fragments or lagging chromosomes during mitosis can be seen as discrete bodies in the cytoplasm of proliferating nucleated cells following exposure to chemical or physical clastogens.

It is clear that bleomycin may exercise its cytotoxic effect through two mechanisms that result in AP and MN. It is also recognised that some chemotheropeutic agents induce their effect at specific stages of the cell cycle (24,25) Therefore the evaluation of BLM activity during different phases of the cell cycle seems essential.

The purpose of this study was to investigate the magnitude of MN formation and AP induction by BLM in normal human peripheral lymphocytes at G0, G1, and G2 phases of the cell cycle.

Material and Methods

Three phases of cell cycle (G0, G1 and G2), three doses of BLM for each phase, and totally 9 groups were used in this study. The cells in each group were subdivided into those used for evaluation of AP and MN with two different time schedules (64 and 96 hrs). For each subgroup, a non-treated control was used. In G0 and G1 groups, the doses of bleomycin were 100, 200 and 300 (g/ml, while for G2 phase lymphocytes they were 50, 100 and 200 (g/ml. All experiments were repeated twice.

* *Micronuclei assay*

The MN assays were done according to the standard in vitro micronucleus test. Blood samples were collected from a healthy normal individual with no history of smoking, exposure to diagnostic X-rays or use of antibiotics for at least three months. 0.5 ml of whole blood was cultured in 4.5 ml RPMI-1640 (Sigma) supplemented with 15% fetal calf serum (FCS), L-glutamine (Sigma), penicillin (100 iu/ml) and streptomycin (100 (g/ml), and PHA-M form (0.1 ml) (Gibco). The method described by Fenech and Morely (26) for MN assay, in which the MN can be scored in cytokinesis blocked lymphocytes at first mitosis, was not used because the experimental design used here required a longer incubation time so that the outcome in terms of MN could be compared with the apoptosis outcome.

For the G0 phase study, the cells were incubated in a CO2 incubator at 37° C and treated with BLM for 4 hrs. BLM was added at the beginning of culture. After treatment, the cells were washed twice with Hank's solution by centrifugation at 1200 rpm for 6 min. Fresh

complete medium containing PHA for stimulation of non-cycling lymphocytes was added to the culture vessels. The cells were cultured until the scheduled evaluation.

For the G1 phase study, cells were incubated with BLM for 4 hrs after stimulation with PHA, then washed by centrifugation and resuspended in complete fresh medium.

For the G2 phase study, PHA stimulated lymphocytes were treated with BLM 4 hrs before harvesting.

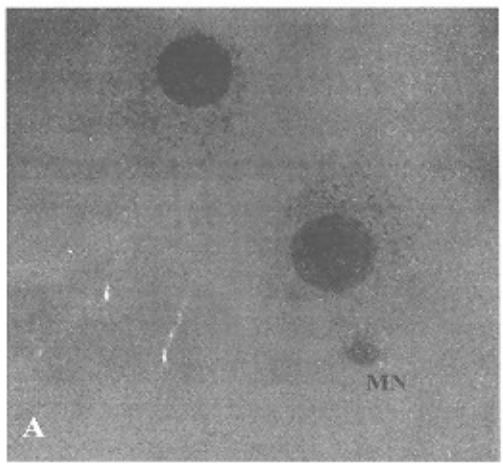
The control cultures were treated according to the above schedules without BLM addition. After either 64 or 96 hrs, the cultures were centrifuged at 1200 rpm for 6 min. The supernatant was removed and cells were exposed to a hypotonic solution (KCl, 0.075 M) for 6 min. at room temperature. After centrifugation and KCl removal, lymphocytes were fixed in Carnoy's fixative consisting of methanol and glacial acetic acid (3:1 v/v) for 20 min., washed 3 times in fixative and slides were prepared. The slides were stained in 10 % Giemsa for 15 min. 1000 cells were analyzed for the presence or absence of micronuclei for each sample.

* *Apoptotic cell assay*

The apoptotic cell assay was done according to Liegler et al.(27). The lymphocytes were isolated from peripheral blood using Ficoll hypaque (provided by Iran blood transfusion organization). Five ml of blood was diluted with RPMI-1640 medium (1:1). To five ml of the diluted blood 3 ml of Ficoll was added and the cells were centrifuged for 20 min at 2000 rpm. The lymphocytes were isolated and washed twice by centrifugation with 2 ml of RPMI 1640 for 7 min. at 1000 rpm. The cells were resuspended in 1 ml RPMI-1640 and cultured. For BLM treatment one million lymphocytes were mixed with 2 ml RPMI-1640 with 15 % FCS and other components mentioned above. Cell cycle phase preparations, BLM treatments and time schedule were as for the MN assay.

Acridine orange/Ethidium bromide double staining was used for fluorescence microscopy of the cells. The stain consisted of 100 μg/ml acridine orange (Sigma) and 100 μg/ml ethidium bromide (Sigma) in phosphate buffer solution (pH=7.2 7.4).1μl of the stain was mixed with 25 μl of cell suspension, transferred to a slide and examined under a fluorescence microscope. A recent comparative study has found this double staining technique to be as reliable as other methods for detecting apoptotic cells (28) and has been used by many investigators (eg. 28-32). In this study 200 cells were analyzed to determine the percentage of apoptotic cells following BLM treatment at different cell cycle stages.

Figure 1 shows a photomicrograph of micronuclei in lymphocytes and apoptotic cells. Data analysis was performed using statistical correlations and the Kruskal-Wallis test.



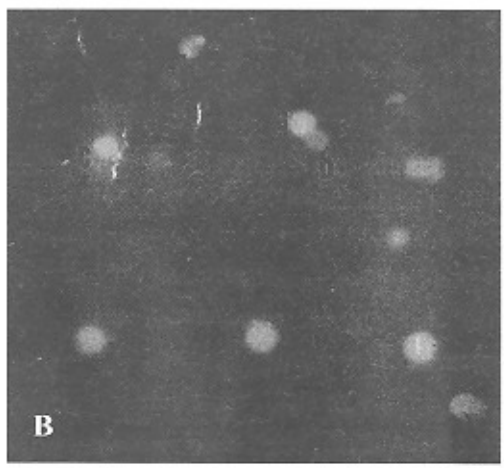


Figure 1: Photomicrographs showing micronucleated (A) and apoptotic (B) lymphocytes. In figure A; (MN) indicates micronuclei and in figure B; (a) indicates Normal, (b) necrotic and (c) apoptotic cells. Magnification of both photomicrographs is x 600.

Results

Figures 2A and 2B show the frequency of micronuclei induced by each dose of BLM in different cell cycle stages after 64 and 96 hrs respectively. As seen, at 64 hr sampling time there is a small but not statistically significant increase in the frequency of MN with increasing dose of BLM for lymphocytes treated at the G0 and G1 phases of the cell cycle. At the same sampling time (64 h) there was a sharp significant ($p<0.05$) increase in the frequency of micronuclei observed for lymphocytes treated with both doses of BLM at the G2 cell cycle stage compared with controls. Comparison of Figures 2A and 2B shows that the incidence of MN decreases with increasing post-treatment sampling time.

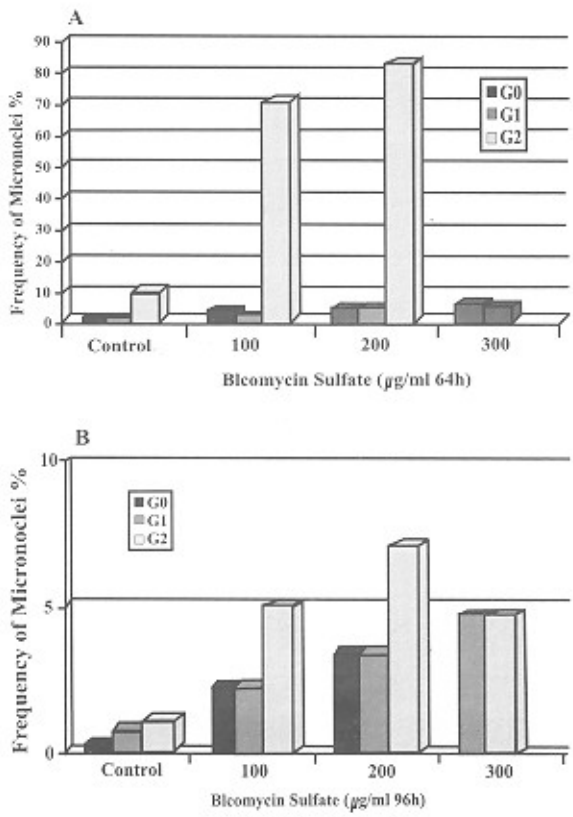


Figure 2: Frequency of micronuclei induced by various doses of bleomycin sulfate in different cell cycle stage. Cells were harvested either at 64 hrs (A) or 96 hrs (B) after BLM treatment. G2 cells weretreated for four hours prior to harvesting.

Figures 3A and 3B show the incidence of apoptosis of lymphocytes treated with different doses of BLM at different cell cycle stages after 64 and 96 hrs respectively. BLM induced a high frequency of apoptotic cells following treatment at G0 and G1 cell cycle stages. The increase in AP was seen to be dose dependent for both G0 and G1 treated cells ($p<0.05$) when harvested at 64 hrs post BLM treatment (Fig 3A). There was not a significant difference between the observed AP cells in G0 and G1 cells treated with similar BLM doses. Frequency of AP cells also increased in G2 cells treated with BLM in a dose dependent manner after 64 hrs sampling time. A similar trend of AP formation was observed for all cell cycle stages studied at 96 hours after BLM treatment (Fig 3B), although the percentage of AP formation was slightly higher in 96 h sampling time compared to 64 h sampling time.

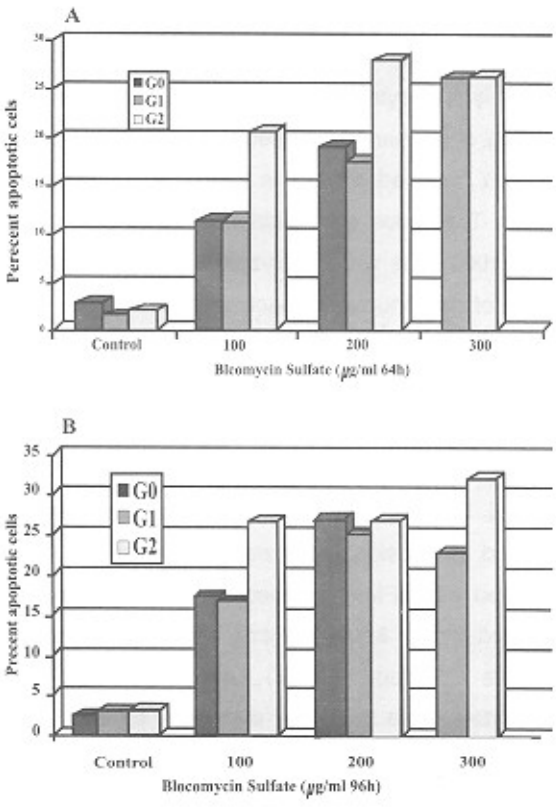


Figure 3: Comparison of the frequency of apoptosis induced by various doses of bleomycin sulfate in different cell cycle stages. Cells were harvested either at 64 hrs (A) or 96 hrs (B) after treatment. G2 cells were treated for four hours before harvesting.

However, at the 96 hr sampling time, there was not a significant difference between 100 and 200 μg/ml BLM in the frequency of AP for cells treated at the G2 cell cycle stage or between 100 and 300 μg/ml BLM for cells treated at the G0 and G1 phases of the cell cycle.

The statistical comparisons between the controls and the treatment groups in both schedules and all the phases, were significantly different ($p<0.05$).

Comparison of Figs 3A and 3B show that there was a small increase in the frequency of AP induction when the sampling time was increased from 64 to 96 hrs. This is in contrast to the effect of sampling time on the incidence of MN.

Discussion

The results show that BLM causes apoptosis and MN formation at 64 and 96 hrs in all the cell cycle stages used in this study (G0, G1 and G2) and are consistent with other studies (16-19) and the report by Logan et al. (33) that manipulation of the cell cycle can enhance the sensitivity to cytotoxic chemicals. The interesting finding that in G2 phase, the cells were more sensitive to bleomycin induced chromosomal damage with MN formation is consistent with the results of other investigators (1, 12, 34). Sikic (35) reported the selectivity of bleomycin on G2 stage of the cell cycle. The lower and similar sensitivity G0 and G1 cells, shown by similar frequency of MN formation, is consistent with another report (36). Bleomycin also induces a higher percentage of apoptosis at G2 phase. Tounekti et al (37) reported fewer cells underwent apoptosis at G0/G1 phases which is consistent with results of this investigation where the percentage of apoptotic cells was lower at both G0 and G1. However,the susceptibility of the cell to the effect of bleomycin at this phase with resultant apoptosis and MN needs to be elucidated particularly in the light of a report by Ishida and Takahashi (38) of the synergistic effect of bleomycin and free radicals on DNA breakage. The reduction in the effect of bleomycin at 96 hrs may be due to activation of recovery mechanisms such as repair enzyme in the case of MN or free radical scavenging in the case of apoptosis (39, 40).

The pattern of dose dependency in apoptosis of this study is consistent with the results of other investigators (2, 14, 41, 42, 43). This may be related to an increase in generation of free radicals with increasing dose of BLM. ROS toxicity to cells is well documented (13, 20, 42). The increase in ROS can induce mitochondrial dysfunction, which causes a shift in the inner mitochondrial trans-membrane potential (44, 45) resulting in release of cytochrome C which activates the caspase pathway (44, 46). Significant increases in the apoptotic cells at G0, G1 and G2 phases were also apparent, and generally are dose dependent. This result is consistent with other investigators who reported that there is apoptosis at G0 (47), G1 (48) and G2 (49). The results of this investigation show that BLM can induce MN and AP simultaneously during the same phases of the cell cycle.

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