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Alkylator-Induced DNA Excision Repair in Human Leukemia CCRF-CEM Cells In Vitro, Measured Using the Single-Cell Gel Electrophoresis (Comet) Assay

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Abstract

The capacity to repair DNA damage is an important factor that affects the therapeutic outcome in cancer treatment. To clarify the cellular repair response, we investigated the kinetics of DNA excision repair initiated by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in human leukemia CCRF-CEM cells at an exponential growth phase in vitro. Using the alkaline single-cell gel electrophoresis (comet) assay, we quantitated the repair kinetics as the amount of DNA single-strand breaks that were generated from the incision and were diminished by the rejoining in the repair process. CEM cells could initiate DNA excision repair in response to BCNU by starting an incision reaction. However, the incision capacity came to a plateau at a concentration of 80 to 100 μ M or after an incubation time of 90 to 120 minutes. When the cells were pulsed with 40 μ M BCNU, the maximal incision occurred at the end of the incubation period, and the repair process was completed within 4 hours. When cells were treated with 100 μ M BCNU, the incised DNA was not rejoined at 4 hours, suggesting that the repair was not completed. Higher concentrations might surpass the cellular capacity for repair and would be associated with increased cell death. Evaluation of the repair process may provide a clue for therapeutic strategies to improve clinical efficacy if accelerated DNA repair is responsible for the drug resistance. *Int J Hematol.* 2002;76:328-332.

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Key words: Alkylating agent; DNA excision repair; Drug resistance; Comet assay

1. Introduction

Alkylating agents constitute a major class of anticancer drugs with well-established activity against hematologic malignancies [1]. These compounds form various cytotoxic alkylations, such as mono-adducts on bases and di-adducts including intrastrand and interstrand cross-links of DNA. The effectiveness of alkylating agents is limited by a number of factors, including drug resistance [2,3]. The capacity to

repair alkylator-induced DNA lesions is an important mechanism underlying drug resistance [2,3]. Such repair mechanisms are classified into 4 general categories: direct repair, base excision repair, nucleotide excision repair, and mismatch repair [3].

Although direct repair with DNA alkyltransferase is important [4], this is not the only enzyme involved in repair because it is incapable of repairing interstrand di-adducts that are crucial to the cytotoxicity [3]. DNA excision repairs including cross-link repairs that can eliminate interstrand cross-links may be more responsible for developing resistance. Therefore, the kinetic evaluation of DNA excision repairs will be useful for clarifying the cellular response to alkylating agents, especially in the context of resistant diseases.

In the present study, we investigated the kinetics of DNA excision repairs initiated by a popular bifunctional alkylator,

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1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), in human leukemia CCRF-CEM cells *in vitro* [5]. The processes of excision repairs initiated by BCNU include incision for removal of the damaged DNA, gap filling by DNA resynthesis, and rejoining by ligation [6]. Therefore, the repair kinetics were quantitated with the alkaline single-cell gel electrophoresis (comet) assay [7] as the amount of DNA single-strand breaks.

2. Materials and Methods

2.1. Chemicals and Reagents

BCNU was purchased from Sigma Chemical Company (St. Louis, MO, USA). The compound was dissolved in 100% ethanol immediately before use.

2.2. Cell Culture

Human leukemia CCRF-CEM cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 2mM L-glutamine. The cells were maintained at 37°C in a humidified atmosphere with 5% carbon dioxide. When the cells were cultured for 8 days, the growth curve was the most log-linear between 24 and 96 hours, suggesting that the cells were in an exponential growth phase during this period. Therefore, subsequent studies were performed with cells in this phase.

2.3. Drug Treatment

CEM cells at 5×10^5 cells/mL were incubated with various concentrations of BCNU for the indicated time periods, followed by washing the cells into fresh media and a subsequent incubation for the indicated time periods.

2.4. The Alkaline Single-Cell Gel Electrophoresis (Comet) Assay

To evaluate the DNA repair kinetics, we performed the alkaline comet assay according to the method previously described but with a slight modification [7]. Approximately 3000 CEM cells after the treatment were mixed with 20 μ L of 0.5% low-melting-point agarose in phosphate buffered saline (PBS) at 37°C. The mixture was layered onto a frosted microscope slide previously coated with 70 μ L of 0.65% normal agarose in PBS, followed by a top layer of 80 μ L of low-melting-point agarose. After solidification, the slide was left in the lysis solution (2.5M sodium chloride, 10mM Tris, 100mM ethylenediamine tetraacetic acid, 10% dimethylsulfoxide, 1% Triton X-100, pH 10) at 4°C for 1 hour. The slide was then placed in the electrophoresis buffer (1mM ethylenediamine tetraacetic acid, 300mM sodium hydroxide, pH 13) for 40 minutes at 4°C to allow the unwinding of DNA. Electrophoresis was conducted for the next 15 minutes at 90 V and 450 mA. After electrophoresis the slide was washed in neutralization buffer (0.4M Tris, pH 7.5) and stained with 25 μ L of 20 μ g/mL of ethidium bromide. One hundred cells per treatment were analyzed by means of the computer-based image-analysis system (Kinetic Imaging Komet Sys-

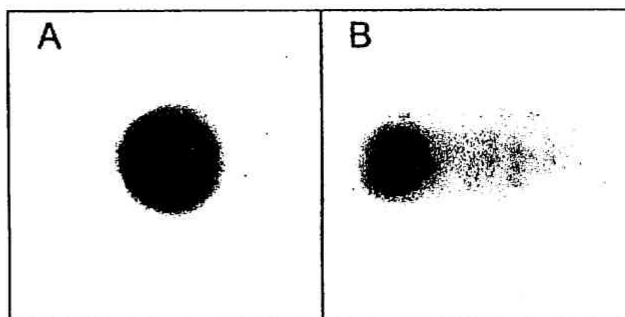


Figure 1. The comet formation. CEM cells were incubated with 100 μ M 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) for 30 minutes, followed by washing the cells into fresh media. The cells were immediately examined by the comet assay. Control (A); the comet generated by treatment with BCNU (B).

tem, Version 4.0, Kinetic Imaging, Ltd., Liverpool, UK). The amount of DNA single-strand breaks was expressed as the "tail moment," which combines measurement of the length of DNA migration and the relative DNA content therein [7].

2.5. Quantitation of Apoptotic Cell Death

To evaluate cytotoxicity, apoptotic cell death was determined by Hoechst staining at 24 hours after the treatment [8]. Cells that had been treated and washed into fresh media were incubated with 2 μ g/mL Hoechst No. 33342 for 30 minutes at 37°C. Nuclei, 200 per treatment condition, were counted under UV illumination. Apoptotic cell death was determined from the nuclear morphology of nuclear condensation and fragmentation.

3. Results

3.1. Comet Formation

A typical comet image is shown in Figure 1. An untreated cell did not exhibit a comet tail, which indicated there were no DNA strand breaks (Figure 1A), whereas a cell treated with BCNU showed comet formation, suggesting that DNA strand breaks had migrated in the electric field (Figure 1B).

3.2. Incision Response to BCNU

To confirm that the comet was generated via the cellular response to BCNU, CEM cells were incubated with various concentrations of BCNU for 30 minutes or with 40 μ M BCNU for various times. The tail moment values increased in both concentration- and time-dependent manners (Figure 2), suggesting an enhanced incision reaction that corresponded to the increased BCNU-induced DNA damage. However, the tail moment value reached a plateau at a concentration of 80 to 100 μ M or after an incubation period of 90 to 120 minutes. Whereas the former result suggests that the incision capacity was saturated, the latter suggests a saturated inci-

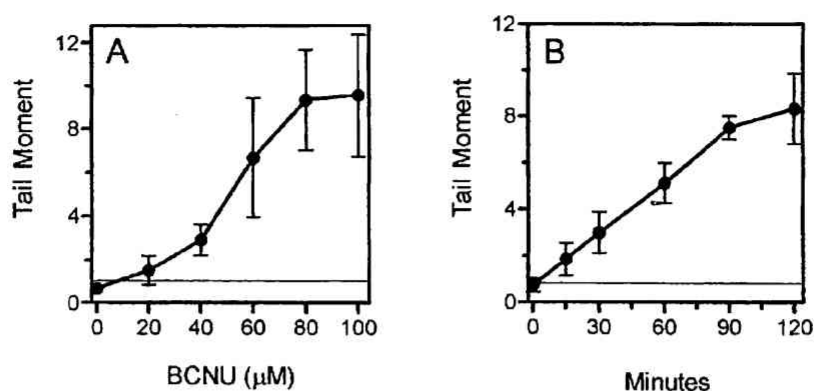


Figure 2. CEM cells were incubated for 30 minutes with various concentrations (0, 20, 40, 60, 80, or 100 μM) of BCNU (A), or with 40 μM BCNU for various times (0, 15, 30, 60, 90, and 120 minutes) (B). After cells were washed into fresh media, they were immediately examined by the comet assay. The tail moment value of the untreated cells was set as a control (hairline). The values are means \pm SD of triplicate determinations. BCNU indicates 1,3-bis(2-chloroethyl)-1-nitrosourea.

sion capacity or the initiation of repair that enabled rejoining of the incised DNA.

3.3. DNA-Repair Process Initiated by BCNU

To determine the kinetics of DNA repair initiated by BCNU, CEM cells were examined by the comet assay at the indicated time points after the cells had been pulsed with BCNU and washed into fresh media. When the cells were treated with 40 μM BCNU, the tail moment was greatest at the end of the incubation period, suggesting the maximal DNA strand breaks resulting from the incision of the repair process (Figure 3A). The tail moment decreased promptly thereafter, suggesting a rapid repair process enabling the rejoining of incised DNA. The tail moment value returned to the control level at 4 hours, representing a successful completion of the process (Figure 3A). The figure also shows that the repair process was most linear between 0 and 2 hours, whereas the slope became lower thereafter. This result sug-

gests that the value of the 2-hour tail moment may be more indicative of completion of the repair process, compared with the value at 4 hours.

When the cells were treated with 100 μM BCNU, the tail moment value was also greatest at the end of the incubation period (Figure 3B). However, in contrast to the result with 40 μM BCNU (Figure 3A), the tail moment did not return to the control level within 4 hours, a result suggesting that repair had not been completed.

3.4. Completion of the Repair Process and the Subsequent Cytotoxicity

To identify the ability to complete the repair process, we incubated CEM cells with various concentrations of BCNU. The tail moment values at 2 hours appeared to increase with concentrations of BCNU beyond 40 μM (60-100 μM), whereas the tail moment generated by lesser concentrations (0-40 μM) of BCNU remained low (Figure 4A). The 100 μM

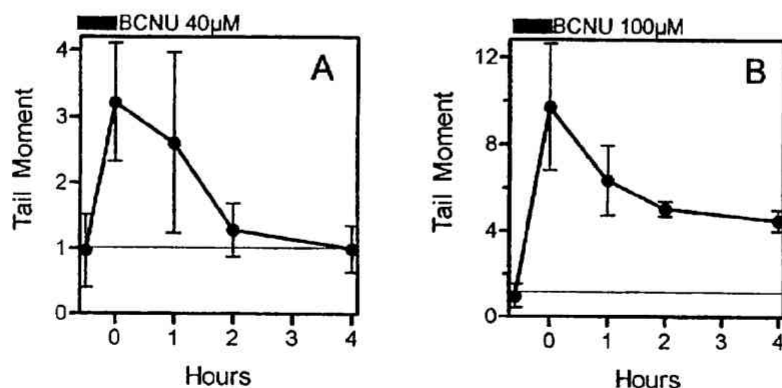


Figure 3. CEM cells were pulsed for 30 minutes with 40 μM (A) or 100 μM (B) of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), followed by washing the cells into fresh media. The cells were then examined by the comet assay at 0, 1, 2, and 4 hours after washing. The tail moment value of the untreated cells was set as a control (hairline). The values are means \pm SD of triplicate determinations.

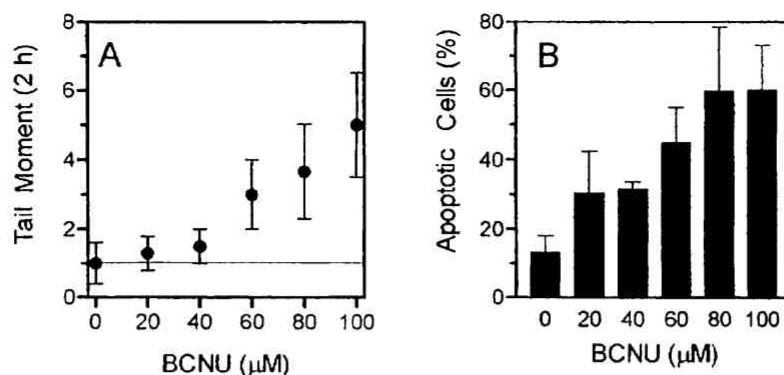


Figure 4. A, CEM cells were incubated for 30 minutes with 1.3-bis(2-chloroethyl)-1-nitrosourea (BCNU) at 0, 20, 40, 60, 80, or 100 μ M, followed by washing the cells into fresh media. The tail moment values were measured 2 hours after washing. The tail moment value of the untreated cells was set as a control (hairline). The values are means \pm SD of triplicate determinations. B, The subsequent cell viabilities were assessed as apoptotic cell death via staining with Hoechst 33342 staining at 24 hours. The values are means \pm SD of triplicate determinations.

concentration was highly toxic to cells, whereas 40 μ M BCNU was minimally toxic (Figure 4B). These results suggest that the higher concentrations surpass the cellular capacity for repair and thereby induce cell death.

4. Discussion

The kinetics of DNA excision repair have been widely investigated in the various contexts of using normal or malignant lymphocytes, normal fibroblasts, or malignant cell lines [9-13]. Lymphocytes can be a paradigm for the investigation of excision repair function, because the cells are quiescent and are not affected by the cell cycle; however, they may not be representative of cycling tumor cells. In addition, the incorporation of thymidine into DNA that has commonly been used to quantitate DNA-repair kinetics may be masked by DNA replication in cycling cells. Thus, it has been difficult to evaluate repair kinetics in the clinically more relevant conditions of actively dividing cells.

In the present study, the comet assay (Figure 1) was successfully used to evaluate the kinetics of alkylator-induced DNA excision repair (Figure 3) in cycling CEM cells in a logarithmic growth phase. Several excision repair processes initiated by DNA alkylation involve the incision and excision of the oligonucleotides that include the damaged nucleotide. Subsequently, there is gap filling by resynthesis of DNA, and ligation rejoins the strands. The comet assay was able to quantitate the first step of the incision and the final step of the rejoining. Previous studies have demonstrated that alkylator-induced DNA lesions do not produce DNA single-strand breaks in the comet assay [12,13]. Therefore, the 0-hour tail moment would appear to reflect the incision step of the DNA-repair process, whereas the decrease in the tail moment thereafter suggests the completion of the repair process by rejoining.

As demonstrated in Figure 3A, malignant CEM cells were able to initiate the repair of DNA damage after alkylation by 40 μ M BCNU. The significant increase in the tail moment observed at the end of the 30-minute incubation period suggested that repair was initiated immediately. The steep

decrease in the tail moment at subsequent times suggested that ligation had occurred and that the repair process was completed quickly.

Conversely, the tail moment value generated by 100 μ M BCNU did not return to the control level at 4 hours (Figure 3B), suggesting that the cells failed to repair the damage induced by this high concentration. At 6 to 8 hours after the end of the incubation period, cells formed a variety of comets; some had almost no comets, and others exhibited larger comet tails (data not shown). Because 50% of the cells treated with 100 μ M BCNU were dead after 24 hours (Figure 4B), the cells that had the larger comet tails at 2 hours might have been induced to die without completing the repair process. In addition, the tail moment at 2 hours generated by 0 to 40 μ M BCNU was minimal, whereas it appeared to increase with concentrations beyond 40 μ M (60-100 μ M; Figure 4A). These results suggest that BCNU at higher concentrations might induce DNA damage that surpasses the cell's capacity to repair it and thereby induce cell death.

In conclusion, with the comet assay we have precisely demonstrated the kinetics of DNA excision repair initiated by BCNU in human leukemia CEM cells *in vitro*. Although BCNU is not used in the treatment of hematologic malignancies, it induces the most common bifunctional alkylations with 90% of mono-adducts and 3% to 5% of interstrand cross-links, effects that are similar to those induced by other alkylators [5]. The agent is commercially available, and it does not need to be metabolized for activation, in contrast to cyclophosphamide. Thus, the repair process initiated by BCNU can be a model for evaluating alkylator-induced DNA excision repair in leukemic cells. Accelerated DNA repair is important to consider in view of chemotherapy. In this regard, the evaluation of the repair process may provide a clue for therapeutic strategies to overcome resistance and improve clinical efficacy.

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