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Enhanced DNA excision repair in CCRF-CEM cells resistant to 1,3-bis(2-chloroethyl)-1-nitrosourea, quantitated using the single cell gel electrophoresis (Comet) assay

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Abstract

Enhanced DNA repair activity is important for the development of cellular resistance to alkylating agents. Here, we quantitated the kinetics of DNA excision repairs initiated by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in human leukemia CCRF-CEM cells. CEM cells that had been established resistant to BCNU (CEM-R) were evaluated in comparison with parental CEM cells (CEM-S). The excision repair kinetics were quantitated as the amount of DNA single strand breaks, which were generated from the incision/excision of the damaged DNA and were diminished by the rejoining of renewed DNA, using the single cell gel electrophoresis (Comet) assay. CEM-R cells were 10-fold more resistant to BCNU than CEM-S cells, and also showed cross-resistance to melphalan and cisplatin. In response to the treatment with BCNU, both CEM-S and CEM-R cells initiated an incision/excision reaction at the end of the incubation period, and completed the rejoining process within 4 hr. While CEM-S cells could not repair the damage induced by the high concentration of BCNU, CEM-R cells completed the repair process regardless of BCNU concentrations, suggesting enhanced excision repairs in CEM-R cells. The excision repair activity of CEM-R cells was increased with regard to the incision reaction and to the rate of the repair. Similar results were obtained using ultraviolet C, suggesting enhanced nucleotide excision repair in CEM-R cells. Thus, the enhanced DNA excision repairs were successfully quantitated in the resistant leukemic cell line using the Comet assay. The evaluation of the repair activity may predict the sensitivity of cancer cells to chemotherapy and provide a clue to overcome the resistance.

Keywords: DNA repair; Alkylating agent; DNA damage; Drug resistance; Acute leukemia; Comet assay

1. Introduction

Alkylating agents are a major class of anticancer drugs for the treatment of various cancers including hematological malignancies [1–3]. The most commonly used agents are analogues of nitrogen mustard (cyclophosphamide, ifosfamide, chlorambucil, melphalan) and nitrosoureas (BCNU, cyclohexylchloroethylnitrosourea, methyl cyclohexylchloroethylnitrosourea) [1–4]. They produce various cytotoxic alkylations such as mono-adducts on O and/or N atoms of bases, and di-adducts including intra- and inter-strand cross-links of DNA. The inter-strand cross-links correlate with the cytotoxicity induced by bi-functional alkylators [1,3,4].

The effectiveness of alkylating agents is limited by a number of factors, including drug resistance [3–6]. A variety of different mechanisms can contribute to the cellular resistance to alkylating agents, among them not only drug export and detoxification, but also efficient DNA repair of the drug-induced alkylation. The capacity to repair alkylator-induced DNA lesions has been suggested associated with the development of drug resistance [3–6]. Such repair mechanisms are classified into four general categories, i.e. direct repair, base excision repair, nucleotide excision repair, and recombinational repair [4].

Among alkylation products, O^6-alkylguanine is directly repaired by a specific suicide protein, O^6-alkylguanine DNA alkyltransferase [7,8]. As the formation of inter-strand di-adducts proceeds through the intermediate pro-

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ara-C, 1-β-D-arabinofuranosylcytosine; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; cisplatin, *cis*-diamminedichloroplatinum (II); UV, ultraviolet C; MTT, methylthiazoletetrazolium; IC_{50}, the concentration to inhibit 50% growth.

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duction of O^6-guanine mono-adducts, the repair through this protein may be important. However, this is not the only enzyme involved in the repair because it is incapable of repairing N^7-alkylguanine mono-adducts or N^7-alkylguanine-N^7-alkylguanine intra-strand di-adducts [3,4]. N-alkylation products are substrates for DNA excision repairs such as base excision repair and nucleotide excision repair. The excision repairs can also remove O-alkylation. More importantly, inter-strand di-adducts are repaired by the combination of nucleotide excision repair and recombination [3,4]. Therefore, the capacity of DNA excision repair may be the most important factor for the development of cellular resistance to alkylators.

If the kinetics of DNA excision repairs can be quantitated in cancer cells, the response to alkylating agents may be predicted especially in the context of resistance. Such an approach may provide a clue to overcome the resistance and to improve clinical efficacy. However, it has been difficult to quantitate DNA repair directly in actively dividing cells because of the lack of appropriate assay methods.

In the present study, we evaluated the kinetics of DNA excision repairs initiated by a popular bi-functional alkylator, BCNU, in human leukemia CCRF-CEM cells *in vitro*. CEM cells resistant to BCNU (CEM-R), which we established here, were evaluated in comparison with parental, sensitive CEM cells (CEM-S). BCNU was chosen because it induces the most common bi-functional alkylation with 90% of mono-adducts and 3–5% of inter-strand cross-links [9]. The excision repair process initiated by BCNU includes incision and excision for removal of the damaged DNA, gap filling by DNA resynthesis, and rejoining by ligation [10–13]. Therefore, the repair kinetics were quantitated as the amount of DNA single strand breaks, which were generated from the incision/excision and were diminished by the rejoining. The alkaline single cell gel electrophoresis (Comet) assay was employed for this quantitation [14–16], as the previous studies suggested its usefulness in both quiescent cells and cycling cells [17–19].

2. Materials and methods

2.1. Chemicals and reagents

BCNU, melphalan, cisplatin, camptothecin, etoposide, ara-C, and F-ara-A were purchased from Sigma. BCNU 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 2 mM L-glutamine. The cells were maintained in a 5% CO_2-humidified atmosphere at 37°.

2.3. Development of BCNU-resistant CEM cells

To develop resistant CEM cells, the parental CEM cells were treated with the escalating concentrations of BCNU. The initial concentration was half the concentration of IC_{50} of CEM cells. The cultures were observed daily and allowed to grow. The drug concentrations for subsequent passages were gradually increased. After 8 months of the drug exposure, the cells grew in the media with 10 μM BCNU. Then, one cell line resistant to BCNU (CEM-R) was cloned by the limiting dilution method.

2.4. Drug treatment and UV exposure

CEM cells (5×10^5 cells/mL) in a logarithmic growth phase were incubated with various concentrations of BCNU, melphalan, cisplatin, camptothecin, etoposide, ara-C, or F-ara-A for the indicated time periods, or exposed to UV at various doses. The cells were then washed into fresh media and subsequently incubated for the indicated time periods.

To evaluate drug sensitivity, MTT assay was used [20]. In brief, 100 μL of CEM cells (5×10^4 cells/mL) were plated and incubated for 24 hr in wells of a 96-well plate. Then various concentrations of each drug were added to the wells. After another 72-hr incubation, 50 μL MTT were added, and the cells were incubated for 4 hr at 37°. The media were removed, and the MTT crystals were solubilized in dimethylsulfoxide. Spectrophotometric absorbance of each sample was measured.

2.5. The alkaline single cell gel electrophoresis (Comet) assay

To evaluate the kinetics of DNA excision repairs, the alkaline Comet assay was performed according to the method previously described with a slight modification [17–19]. Approximately 3000 CEM cells after the treatment were mixed with 20 μL of 0.5% low melting point agarose in PBS at 37°. The mixture was layered onto a frosted microscope slide previously coated with 70 μL of 0.65% normal agarose in PBS, followed by a top layer of 80 μL of the low melting point agarose. After solidification, the slide was left in the lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM ethylenediamine tetraacetic acid, 10% dimethylsulfoxide, 1% Triton X-100, pH 10) at 4° for 1 hr. The slide was then placed in the electrophoretic buffer (1 mM ethylenediamine tetraacetic acid, 300 mM NaOH, pH 13) for 40 min at 4° to allow unwinding of DNA. Electrophoresis was conducted for the next 15 min at 90 V and at 450 mA. After the electrophoresis, the slide was washed in neutralization buffer (0.4 M Tris, pH 7.5) and stained with 25 μL of 20 μg/mL of ethidium bromide. One hundred cells per treatment were analyzed using the computer-based image analysis system (Kinetic Imaging Komet system, Ver. 4.0). The amount of DNA single strand

breaks was quantitated and expressed as the "tail moment," which combined a measurement of the length of the DNA migration and the relative DNA content therein [14].

2.6. Quantitation of apoptotic cell death

To evaluate cytotoxicity, apoptotic cell death was determined by Hoechst staining at 24 hr after the treatment [21]. Cells having been treated and washed into fresh media were incubated with 2 μg/mL Hoechst No. 33342 for 30 min at 37°. Nuclei, 200 per treatment condition, were counted under UV illumination. Apoptotic cell death was determined from the nuclear morphology of nuclear condensation and fragmentation.

Table 1
Drug sensitivities of CEM-S and CEM-R cells

Drugs	IC_{50} (μM)		Relative resistance
	CEM-S	CEM-R	
BCNU	2.4	24	10.0
Melphalan	0.9	7	7.8
Cisplatin	0.8	5	6.3
Camptothecin	0.02	0.045	2.3
Etoposide	4	6	1.5
ara-C	0.8	0.9	1.1
F-ara-A	13	22	1.7

Both the sensitive (CEM-S) and the resistant (CEM-R) cells were incubated with various concentrations of anticancer agents for 72 hr. The IC_{50} was then determined using MTT assay. Relative resistance values were obtained by dividing the IC_{50} value of CEM-R cells by that of CEM-S cells.

3. Results

3.1. Establishment of BCNU-resistant CEM cells (CEM-R)

To determine drug sensitivity, parental, sensitive CEM cells (CEM-S) and BCNU-resistant CEM cells (CEM-R) were incubated for 72 hr with various concentrations of BCNU, and then applied to MTT assay. The IC_{50} was extrapolated in each cell line (Fig. 1A). CEM-R cells were 10-fold more resistant to BCNU than CEM-S cells. The level of the resistance would be close to that found in clinic [3].

To determine the cytotoxicity of BCNU, both CEM-S and CEM-R cells were incubated for 30 min with various concentrations of the drug, followed by washing into fresh media and by a subsequent incubation for 24 hr. Apoptotic cell death was increased in number in CEM-S cells by the escalating concentrations of BCNU whereas the cytotoxicity was minimum in CEM-R cells (Fig. 1B). The subsequent Comet assay was conducted under the 30-min incubation with BCNU while the drug sensitivity was determined using the continuous incubation for 72 hr. The levels of cytotoxicity were different between these two conditions.

3.2. Cross-resistance in CEM-R cells

To determine cross-resistance, CEM-R cells were incubated with various anticancer agents, and then applied to MTT assay (Table 1). The cells exhibited the cross-resistance to a similar alkylating agent, melphalan, and a platinum analogue, cisplatin. The cells did not show resistance against a topoisomerase II inhibitor, etoposide, nor nucleoside analogues such as ara-C and F-ara-A.

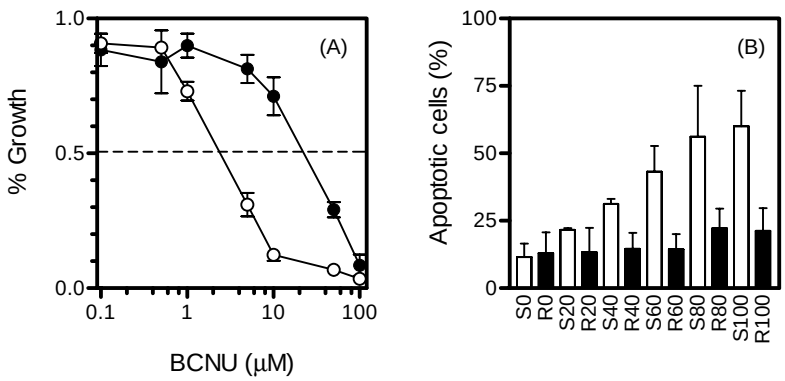

Fig. 1. (A) Establishment of BCNU-resistant CEM (CEM-R) cells. Parental CEM cells (CEM-S) were maintained with BCNU. One cell line resistant to BCNU (CEM-R) was cloned by the limiting dilution method. Both CEM-S (○) and CEM-R cells (●) were incubated for 72 hr with various concentrations (0, 0.1, 0.5, 1, 5, 10, 50, and 100 μM) of BCNU. The cell growth was then determined by MTT assay. The values are the means ± SD of triplicate determinations. The IC_{50} was extrapolated in each cell line. CEM-R cells were 10-fold more resistant to BCNU than CEM-S cells. (B) Cytotoxicity of BCNU in both CEM-S (open bar) and CEM-R cells (closed bar). Both cells were incubated for 30 min with various concentrations (0, 20, 40, 60, 80, or 100 μM) of BCNU, followed by washing the cells into fresh media. Cytotoxicity was determined as apoptotic cell death using Hoechst No. 33342 staining at 24 hr after the cells had subsequently been incubated in fresh media. The values are the means ± SD of triplicate determinations. S0, CEM-S cells untreated; S20, CEM-S cells treated with 20 μM BCNU; S40, CEM-S cells treated with 40 μM BCNU; S60, CEM-S cells treated with 60 μM BCNU; S80, CEM-S cells treated with 80 μM BCNU; S100, CEM-S cells treated with 100 μM BCNU; R0, CEM-R cells untreated; R20, CEM-R cells treated with 20 μM BCNU; R40, CEM-R cells treated with 40 μM BCNU; R60, CEM-R cells treated with 60 μM BCNU; R80, CEM-R cells treated with 80 μM BCNU; R100, CEM-R cells treated with 100 μM BCNU.

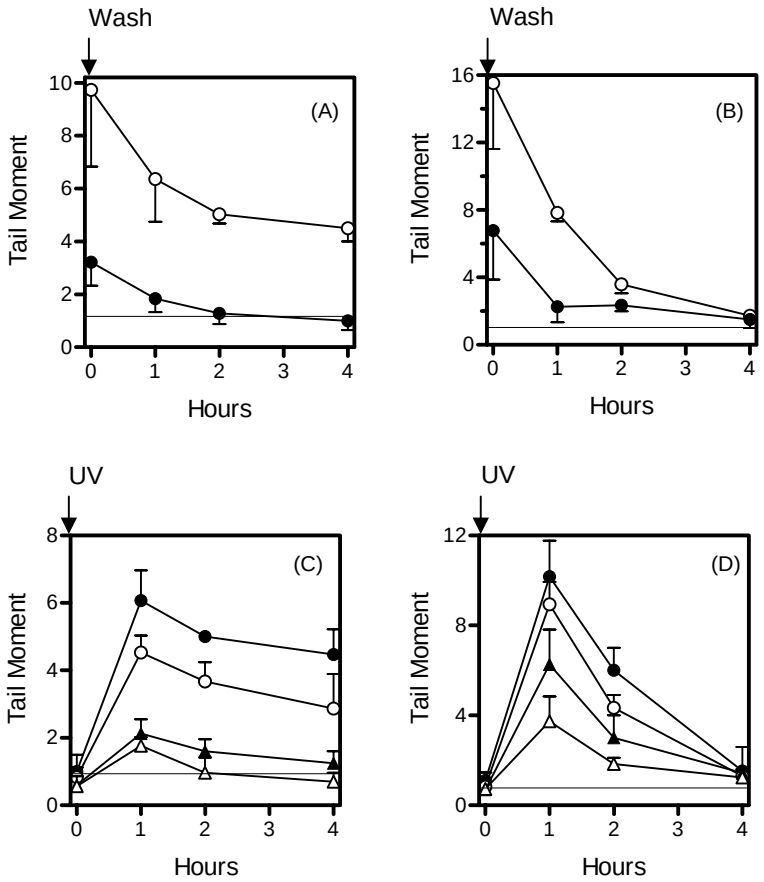

Fig. 2. (A and B) Time course of DNA excision repairs in CEM cells initiated by BCNU. Both CEM-S (A) and CEM-R cells (B) were pulsed for 30 min with 40 μM (●), or 100 μM (○) of BCNU, followed by washing the cells into fresh media. The cells were applied to the Comet assay at 0, 1, 2, and 4 hr after washing. (C and D) Time course of nucleotide excision repair in CEM cells initiated by UV. Both CEM-S (C) and CEM-R cells (D) were exposed to UV at 5 J/m^2 (△), 10 J/m^2 (▲), 20 J/m^2 (○), or 50 J/m^2 (●). The cells were applied to the Comet assay at 0, 1, 2, and 4 hr after irradiation. The tail moment value of the untreated cells was set as a control (hairline). The values are the means ± SD of triplicate determinations.

3.3. Time course of DNA excision repairs in CEM cells initiated by BCNU

To determine the kinetics of DNA excision repairs initiated by BCNU, both CEM-S and CEM-R cells were applied to the Comet assay at the indicated time points after the cells had been pulsed with BCNU (Fig. 2A and B). When CEM-S cells were treated with a minimally toxic concentration (40 μM) of 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 (Fig. 2A). The tail moment was decreased promptly thereafter, suggesting a rapid rejoining of the incised DNA. The tail-moment returned to the control level at 4 hr, representing successful completion of the repair process. When the cells were treated with a highly toxic concentration (100 μM) of BCNU, the tail moment was also greatest at the end of the incubation period (Fig. 2A). However, in contrast to the result of 40 μM BCNU, the tail moment did not return to the control level within 4 hr, suggesting that the repair was not completed. In both concentrations, the repair process was most linear between 0 and 1 hr, whereas the slope became less thereafter. This suggests that the value of the 0/1 hr tail moment ratio represented the rate of the repair (Table 2).

When CEM-R cells were incubated with BCNU, the tail moment at the end of the incubation period was higher than that produced in CEM-S cells, suggesting an enhanced incision reaction in the resistant cells. The tail moment returned to the control level within 4 hr regardless of the drug concentration (Fig. 2B). The rates of the repair were greater in CEM-R cells than those in CEM-S cells (Table 2), suggesting an accelerated rate of BCNU-induced excision repairs in CEM-R cells.

3.4. Incision reaction in CEM cells initiated by BCNU

To confirm that the tail moment was generated through the cellular response to BCNU, both CEM-S and CEM-R cells were incubated with various concentrations of BCNU, and immediately applied to the Comet assay. In CEM-S cells, the tail moment values were increased in a concentration-dependent manner (Table 2), suggesting an incision reaction corresponding to the increased BCNU-induced DNA damage. However, the tail moment value reached a plateau at concentrations of 80–100 μM, sug-

Table 2
DNA excision repair activity of CEM-S and CEM-R cells

		CEM-S	CEM-R	*P* values
BCNU-induced excision repair	BCNU (μM)			
Incision activity	20	1.5	2.7	0.04
	40	2.9	6.8	0.01
	60	6.7	10.5	0.04
	80	8.7	13.0	0.04
	100	9.4	15.6	0.04
Rate of repair	40	1.5	2.5	0.12
	100	1.5	2.0	0.04
Nucleotide excision repair	UV dose (J/m^2)			
Incision activity	1	1.7	1.1	0.3
	5	1.9	3.7	0.04
	10	2.1	6.3	0.02
	20	4.5	8.9	0.003
	50	6.1	10.1	0.02
	100	7.4	13.7	0.01
Rate of repair	5	1.8	2.0	0.2
	10	1.4	2.0	0.16
	20	1.4	2.0	0.03
	50	1.2	1.8	0.04

Both the sensitive (CEM-S) and the resistant (CEM-R) cells were incubated with BCNU for 30 min or irradiated with UV, washed into fresh media, and applied to the Comet assay at 0, 1, 2 and 4 hr, thereafter. Incision activity represented the 0-hr tail moment for BCNU treatment or the 1-hr tail moment for UV treatment. The rate of the repair represented the tail moment ratio between 0 and 1 hr for BCNU treatment or the ratio between 1 and 2 hr for UV treatment.

gesting that the incision capacity was saturated. In CEM-R cells, the tail moment was also increased by the escalating concentrations of BCNU, but it did not reach a plateau. The levels of the tail moment in CEM-R cells were significantly greater than those generated by the respective concentrations of BCNU in CEM-S cells (Table 2). These results suggest an enhanced incision activity of BCNU-induced excision repairs in CEM-R cells.

3.5. Time course of nucleotide excision repair in CEM cells initiated by UV

While alkylating agents initiate base excision repair and nucleotide excision repair, UV induces nucleotide excision repair exclusively. To evaluate the capacity of nucleotide excision repair, both CEM-S and CEM-R cells were exposed to UV at various doses, then applied to the Comet assay (Fig. 2C and D). When CEM-S cells were exposed to UV, the tail moment values were greatest at 1 hr after irradiation, suggesting that the maximal incision reaction occurred. The tail moments were gradually decreased, suggesting that the subsequent repair process enabled rejoining of the incised DNA (Fig. 2C). At higher doses, the tail moment did not return quickly. As the strand breaks generated by the incision step remain open until the rejoining is completed by ligase I, the results also suggest that some sensitive cells might be deficient in ligase I. The rates of the repair were also measured, using the ratio of 1/2 hr tail moment values here (Table 2).

When CEM-R cells were exposed to UV, the tail moment values at 1 hr after irradiation were higher than those generated in CEM-S cells, suggesting an enhanced incision reaction in the resistant cells (Fig. 2D). The tail moment values returned to the control level at 4 hr regardless of the dosage of UV. The rates of the repair were greater than those in CEM-S cells (Table 2), suggesting an accelerated rate of nucleotide excision repair in CEM-R cells.

3.6. Incision reaction in CEM cells initiated by UV

To confirm the incision reaction in the process of nucleotide excision repair, both CEM-S and CEM-R cells were exposed to different doses of UV, and the samples were applied to the Comet assay at 1 hr after irradiation. The tail moment values were increased in a dose-dependent manner in both cell lines, suggesting an incision reaction corresponding to the increased UV-induced damage (Table 2). However, the tail moment values were significantly greater in CEM-R cells than those in CEM-S cells (Table 2). These results suggest an enhanced incision activity of nucleotide excision repair in CEM-R cells.

4. Discussion

DNA repair has widely been investigated to seek for new therapeutic strategies of cancer [22–26], as increased repair activities are closely associated with drug resistance [3–5]. Quiescent normal lymphocytes and fibroblasts at a confluent phase can be used for investigation of DNA repair because the cells are not affected by the cell cycle. These cells can mimic slow-growing tumors or a dormant population in tumor tissues, however, they may not represent cycling malignant cells. Moreover, the cellular activities such as DNA repair might be reduced in the cells at a confluent state. In addition, the measurement of thymidine incorporation, which is commonly used to quantitate the kinetics of DNA repair, might be masked by DNA replication in cycling cells. Therefore, it has been difficult to evaluate the repair kinetics as a whole cell in actively dividing cells that are clinically more relevant.

Our previous studies have suggested the usefulness of the Comet assay for quantitating DNA repair kinetics in both quiescent and cycling cells [17–19]. The present study successfully quantitated the increased repair activity in the resistant leukemic cells in comparison with the sensitive counter part using the Comet assay. Several excision repair processes initiated by BCNU consist of incision and excision of the damaged nucleotide, gap filling by resynthesis of DNA, and rejoining by ligation. Therefore, the first step of the incision and the final step of the rejoining were measured as amounts of DNA single strand breaks.

Both DNA damage induced by BCNU and the cellular responses to such insults are complex. The strand breaks detected by the Comet assay could be results of direct strand breaks induced by BCNU, alkali-labile sites, or the incision reaction by repair enzymes at sites of DNA damage. Single strand breaks are directly induced by BCNU due to alkylation of sugar-phosphate backbone of DNA (alkylphosphotriesters) or due to alkylation of bases. The phosphoryl oxygens of the sugar-phosphate backbone are alkylated and cause alkylphosphotriesters. They undergo hydrolysis, leading to strand breakage. However, the process of hydrolysis that causes strand scissions is very slow [1]. Moreover, alkylphosphotriesters are highly persistent DNA modifications that stay in the DNA for 20–50 hr [27]. Since BCNU-induced strand breaks detected by the Comet assay disappeared almost within 4 hr, it would seem unlikely that such long-lived alkylphosphotriesters and the slow reaction of hydrolysis contribute significantly to DNA single strand breaks in the present experimental setting. Among BCNU-induced alkylation, base adducts are labile. Such alkali labile base damages are usually repaired by excision repairs, which is evaluated by the Comet assay. Alternatively, alkali labile bases can produce abasic sites, resulting in single strand breaks by the action of endonucleases [1]. But, in the previous study, the Comet assay detected the increase in the amount of single strand breaks of the alkylator-treated cells when the cells were further incubated with endonuclease [28]. This suggests that such abasic sites are unlikely to produce strand scissions without the exogenous endonuclease in the Comet assay procedure. The addition of alkaline solution during the assay might also result in a break at the abasic sites. However, it has been reported that the abasic sites formed by ethyl nitrosourea at concentrations below 2 mM did not produce single strand breaks during the assay procedure [29]. We have attempted to further minimize this slight possibility by carrying out alkali unwinding as well as the electrophoresis step at 4° in the experiment. Given these backgrounds, the labile sites would be unlikely to produce significant single strand breaks under the present experimental condition. Thus, the excision repair kinetics were precisely evaluated in actively dividing CEM cells using the Comet assay, reflecting the initial rise of the tail moment as the incision step, and the subsequent diminution as the rejoining process [17–19].

The Comet assay characterized the BCNU-resistant CEM cell line (CEM-R) from the viewpoint of the capacity of DNA excision repair, in comparison with the parental, sensitive CEM (CEM-S) cells. Both CEM-S and CEM-R cells could respond to BCNU by initiating DNA excision repairs (Fig. 2A and B). When CEM-S cells were treated with 40 μM BCNU, the increase in the tail moment observed at the end of the incubation period suggested an immediate initiation of the incision reaction (Fig. 2A). Subsequently, the tail moment was decreased with time, suggesting the rejoining of the incised DNA by ligation. The tail moment returned to the control level within 4 hr, suggesting completion of the repair process (Fig. 2A). When CEM-R cells were treated with 40 μM BCNU, the tail moment was elevated higher at the end of the incubation period, and was decreased more quickly thereafter, compared with CEM-S cells (Fig. 2B, Table 2). CEM-R cells completed the repair process at 4 hr regardless of the concentration of BCNU whereas CEM-S cells could not repair the damage induced by the high concentration (100 μM) of BCNU. Similar results were also obtained using UV (Fig. 2C and D, Table 2). Thus, these findings suggest that the DNA excision repair function including nucleotide excision repair was enhanced in CEM-R cells with regard to the incision activity and to the rate of the repair (Table 2).

The area under the curve of the tail moment for the low-dose (40 μM) BCNU in CEM-S cells was smaller than that for CEM-R cells (Fig. 2A and B), which seemed suggestive of a smaller repair capacity or fewer BCNU-induced lesions. However, the Comet assay only measures the amount of DNA single strand breaks. The amount of single strand breaks is not necessarily proportional to the amount of BCNU-induced lesions since the breaks occur mainly through the repair process. Moreover, initial rise of the tail moment depends on the activity of the incision step. If the incision reaction is enhanced, the level of the tail moment becomes higher, leading to the greater area under the curve of the tail moment. Conversely, the subsequent decrease in the tail moment suggests the rejoining process in the repair. If the repair function is hyperactive, the tail moment decreases more quickly, leading to the smaller area under the curve. Therefore, it would be difficult to directly relate the area under the curve of the tail moment to the repair function or to BCNU-induced lesions.

The formation of DNA inter-strand cross-links by BCNU retard the migration of the fragmented DNA in the Comet assay. It might influence the results in the two ways. First, the decrease in the tail moment with time could also be related to the development of cross-links. Second, the cross-link formation could make the area under the curve of the tail moment smaller in the sensitive cells that did not repair the cross-links. The Comet technique cannot rule out such possibilities. However, the cross-links occupy less than 5% among all the adducts formed by BCNU. Moreover, the time course of single strand breaks induced by BCNU (Fig. 2A and B) was very similar to the kinetics induced by UV that did not induce cross-links (Fig. 2C and D). Therefore, the influence of cross-links on the results might be minimal.

As mentioned above, the results obtained in the context of UV (Fig. 2C and D) suggest the enhanced capacity of nucleotide excision repair. In the process of nucleotide excision repair, the initial step is recognition of DNA damage by the XPA protein and RPA. These proteins then recruit transcription factor TFIIH that contains helicase activity. Next, ERCC1-XPF and XPG cut the DNA on either side of the damage. This is followed by excision of

the damaged oligonucleotide fragment containing the lesion, and then by DNA synthesis to fill the excision gap using DNA polymerase. Finally, joining of the nick by ligation probably using DNA ligase I completes the process [13]. Since the present study focused on the kinetics of excision repairs, neither the gene expression nor the protein level for these repair enzymes were determined. However, the increased incision reaction and the accelerated repair rate in CEM-R cells might be associated with the increased activities of repair enzymes such as ERCC1, DNA polymerase, and DNA ligase, which were previously suggested involved in the mechanisms of drug resistance [5,30,31].

Cancer cells develop drug resistance by the multifactorial nature. Decreased accumulation, increased efflux, increased inactivation by glutathione, by glutathione-*S*-transferase, or by metallothionein, altered metabolism, and increased tolerance of unrepaired damage may all contribute to the resistance [1,4]. It appears likely that DNA repair also contributes to resistance to bi-functional chemotherapeutic agents [1,3–5]. Many cisplatin-resistant cell lines have increased repair activity, suggesting an increase in nucleotide excision repair. O^6-Alkylguanine-alkyltransferase-mediated DNA repair is an important mechanism of resistance to nitrosoureas and related alkylating agents in many cell lines. As the formation of cytotoxic inter-strand di-adducts proceeds through the intermediate production of O^6-guanine mono-adducts, the repair through this protein may be crucial. In some cell lines, resistance appears to be determined not only by the alkyltransferase levels but also by possible contributions of both nucleotide- and base-excision repairs. Increased repair of inter-strand cross-links is also found in some nitrogen mustard-resistant cells, suggesting the enhanced nucleotide-excision/recombination-repair pathway [4]. Thus, multiple mechanisms of cellular resistance would occur in a given cancer cell population.

As to CEM-R cells in the present study, the excision repair function was increased only 1.5–2-fold with regard to the incision activity or to the rate of repair despite the 10-fold resistance measured by the IC_{50} value. As described above, various mechanisms contribute to the induction of the resistance. Enhanced excision repair might not be the only mechanism for the development of the resistance in CEM-R cells. Nevertheless, CEM-R cells could repair the DNA damage and survive the high concentration (100 μM) of BCNU, suggesting a close association between the repair capacity and the drug resistance. This would be also supported by the cross-resistance to melphalan and cisplatin (Table 1). Since the level of the resistance (10-fold) in CEM-R cells was close to that found in clinic, the enhanced repair capacity described here would be encountered in chemoresistant leukemic cells in clinical settings. Therefore, the evaluation of the excision repair capacity may predict the sensitivity of cancer cells to chemotherapy and provide a clue for new therapeutic strategies to overcome the resistance.

In conclusion, it has been difficult to evaluate directly DNA repair in resistant cancer cells because of the lack of appropriate assay methods. The present study has clearly demonstrated the enhanced activity of DNA excision repairs in the resistant leukemia cell line using the Comet assay.

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