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Reduced DNA ligase activity in etoposide resistant human lymphatic leukaemia CEM cells

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Abstract

Drug resistance is an obstacle preventing success of cancer chemotherapy. Resistance of vaccinia virus towards the topoisomerase II (topo II) targeting anti-cancer drug etoposide has been mapped to the viral DNA ligase gene. The present study was performed to elucidate if the DNA ligase activity, besides topo II levels, was altered in human lymphatic leukaemia cell strains with different levels of etoposide resistance. At measurements of DNA ligase activity with specific substrates, to distinguish between different DNA ligases, a reduced DNA ligase activity was observed in the resistant substrains. In contrast, the initial step of the ligation process, formation of DNA ligase–AMP complex, did not decrease in the resistant cell strains, suggesting an alteration in a later reaction leading to a deteriorated DNA ligation. The results suggest that decreased DNA ligase activity, besides topo II alterations, may contribute to etoposide resistance of the investigated CEM cells. The relevance of this finding will be further investigated.

Keywords: Etoposide; Topoisomerase II (EC 5.99.1.3); DNA ligase (EC 6.5.1.1); Drug resistance; Leukaemia; CCRF-CEM cells

1. Introduction

Etoposide is a potent chemotherapeutic agent frequently used against a spectrum of malignancies, both haematological diseases and solid tumours. Drug resistance is considered one of the greatest problems in the treatment of cancer, and many different biochemical and cytogenetic alterations can be involved in the development of a drug resistant cell ([1], review). One mechanism is the alteration or down regulation of a cellular target, such as the nuclear enzyme topo II [2]. Topo II is the target of a number of clinically important anti-cancer drugs; e.g. the antracyclines, the podophyllotoxin derivatives, amsacrine [3] and mitoxantrone [4]. Its function is to regulate the topological state of DNA during replication, ligation, transcription and recombination ([5], review). The unknotting of double stranded DNA is a complicated process involving binding, cleavage, and passage of the uncleaved DNA double strand and subsequent ligation of the strand break. The drug acts by binding to topo II and prevents the religation of the cleaved double strands, thus inducing accumulation of topo II-drug–DNA complex. The subsequent events leading to cell death are not completely revealed. It has been shown that cell lines resistant to topo II targeting drugs have reduced levels or activity of topo II or shows point mutations of the drug binding region of the enzyme ([6], review).

A previous study revealed that vaccinia virus telomer resolution, occurring at the late phase of DNA replication, is blocked by the topo II targeting anti-cancer drug etoposide [7]. After isolation of etoposide resistant viruses mutations conferring resistance were mapped to the viral DNA ligase gene, and not as expected to the topoisomerase gene. The investigators also showed that deletions in the vaccinia ligase gene also conferred etoposide resistance. Reinsertion of wild-type or mutant DNA ligase in the viral thymidine kinase locus confirmed viral DNA ligase dependent sensitivity to etoposide and also to another topo II

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Abbreviations: Topo II, topoisomerase II; DTT, dithiothreitol.

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interacting agent, amsacrine. A comparison of the peptide sequences of vaccinia DNA ligase with the amino sequences of other DNA ligases revealed that vaccinia DNA ligase is more related to the DNA ligase III than to mammalian DNA ligase I or IV [8,9].

If altered DNA ligase activity confers resistance to etoposide also in human cancer cells such alterations in patients may consequently cause impairment of treatment outcome. Furthermore, topo II–DNA complex formation alone would not explain the mechanism of action of the drug, and DNA ligase has to be considered a second drug target. Therefore, this study was performed to elucidate if the DNA ligase activity was altered in human lymphatic leukaemia cell strains CEM/VP100, CEM/VP200, CEM/VP500 with different levels of etoposide resistance, as compared with their sensitive parent cell line CCRF-CEM.

2. Materials and methods

2.1. Cell culture and culture conditions

Etoposide resistant cell substrains, CEM/VP100, CEM/VP200 and CEM/VP500 were derived by treatment of the acute lymphatic leukaemia cell line CCRF-CEM with increasing etoposide, VEPESID®, concentrations of 100, 200 and 500 ng/mL and were grown in the absence of etoposide for 2 weeks before harvest. The cells were grown in suspension cultures in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum in 5% CO_2 at 37°. The cells were routinely tested for mycoplasma by staining with Hoechst dye [10] or by mycoplasma culture test and were free from infection.

2.2. Preparation of cell extracts

For preparation of nuclei, 100×10^6 cells were used. Two separate preparations of each cell strain were made. After centrifugation, the medium was aspirated and the cells were washed twice with ice cold PBS. The cells were suspended in 3 mL ice cold PBS containing 5 mM EDTA and incubated on ice for 1 hr. After centrifugation at 1500 *g* for 4 min, the supernatant was aspirated. The cells were resuspended in 3 mL ice-cold Schibler buffer containing 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES (pH 7.5), 0.3 M sucrose, 0.8% (v/v) NP40 and 0.5 mM DTT, transferred to a Dounce homogeniser (KEBO) and disrupted by 20 strokes with pestle A. Nuclei were washed in 50 mL of 5 mM $MgCl_2$, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 20 mM HEPES (pH 7.5), 1 mM DTT and 20% (v/v) glycerol was added followed by centrifugation at 1000 *g* for 8 min. The supernatant was aspirated and protease inhibitors, aprotinin (2 mg/mL), phenylmethylsulfonyl fluoride (PMSF) (1 mM), Leupeptin (0.5 μg/mL), Pepstatin (0.5 μg/mL), Chymostatin (0.5 μg/mL), and *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (0.5 μg/mL) were added. All manipulations were performed on ice. High-salt extraction buffer (0.5 M NaCl, 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 1 mM EDTA and 10% (v/v) glycerol), 200 μL, was added to the cell nuclear extract and the nuclei were disrupted by sonication (Soniprep 150, MSE), 5×5 s, at 1-min intervals. The nuclear extract was placed on ice for 1 hr, followed by centrifugation at 10,000 *g* for 30 min at 4°. To remove the nucleic acids the supernatant was transferred to 1 g DEAE cellulose (DE52, Whatman), pre-equilibrated in extraction buffer, and incubated on ice for 1 hr. After occasional rotation of the tube on ice, the tube was centrifuged at 10,000 *g* for 30 min at 4°, and the supernatant was collected. For preparation of whole cell extract, exponentially growing cells (3×10^6) were solubilised in 50 μL of high-salt extraction buffer, incubated on ice for 15 min, frozen in liquid N_2 and thawed at one cycle. After centrifugation at 10,000 *g* for 30 min at 4°, the supernatant was collected. The protein concentration was determined with the COOMASSIE® Protein Assay Reagent (Pierce).

2.3. DNA ligase assays

Reaction mixture (60 μL) contained 60 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP and 50 μg/mL of bovine serum albumin, polynucleotide substrate [^{32}P]oligo(dT)·poly(dA) or [^{32}P]oligo(dT)·poly(rA) (10,000–20,000 cpm) [11] and a limiting amount of DNA ligase. After incubation for 30 min at 16°, the reaction was terminated by heating to 85° for 10 min and placed on ice for 10 min. Bacterial alkaline phosphatase (0.2 U) (Sigma) was added to each sample and were further incubated to 85° for 10 min. This step was repeated once. After addition of 40 μL of 1 mg/mL BSA and precipitation with 500 μL of 20% (w/v) trichloroacetic acid (TCA), the acid-precipitable material was collected onto nitrocellulose filters (0.45 μm, ME25) (Schleicher & Schuell). The filters were washed with 10% (w/v) TCA, dried and the radioactivity was determined. T4 DNA ligase (New England Biolabs) was used as a positive control.

2.4. DNA ligase–AMP complex formation

The assay is based on the ability of DNA ligases to react with ATP to covalently form a DNA ligase–AMP complex. DNA ligase was adenylylated in 10 μL reaction mixture containing 60 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 50 μg/mL BSA and 0.5 μCi α-[^{32}P]ATP (3000 Ci/mmol) (Amersham) at room temperature for 15 min. The reaction was terminated by the addition of 2 μL of SDS sample buffer and samples were heated at 90° for 10 min. Proteins were separated by 7.5% (w/v) SDS-polyacrylamide gel electrophoresis [12]. Gels were fixed in 10% (w/v) acetic acid for 10 min, dried under vacuum and exposed to a film (Fuji medical X-ray film) overnight

at −80°, and the enzyme–adenylate complexes were detected by autoradiography. ^{14}C-Labelled proteins and Rainbow markers (Amersham) were used as molecular weight markers.

2.5. Western blot analysis

Proteins were separated by denaturing SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell) in 25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3). Membranes were blocked for 2 hr at room temperature in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST) and 5% (w/v) dried milk, followed by incubation over night at 4° with antibodies diluted in TBST plus 1% (w/v) bovine serum albumin. After removal of unbound antibodies the membranes were incubated for 2 hr at room temperature with horse radish peroxidase (HRP) labelled secondary antibodies and the antigen–antibody complexes were detected by enhanced chemiluminescence (Amersham). For DNA ligase I detection the following procedure was used: after incubation with anti-DNA ligase I antibody (overnight, 4°), the membrane was firstly incubated with biotinylated anti-mouse Ig (1 hr, room temperature) and then with streptavidin-horseradish peroxidase conjugate. Finally, the antigen–antibody complex was detected with the ECL system (Amersham). The antibodies used were polyclonal rabbit antibodies raised against DNA ligase III (generously provided by Professor T. Lindahl, Imperial Cancer Research Fund, Clare Hall Laboratories, London) and monoclonal antibody (clone 7A12, Serotec Ltd.) against DNA ligase I. Topo IIα and IIβ were determined according to Zhou *et al.* [13].

3. Results

3.1. Measurements of DNA ligase activity

DNA ligase activity was measured in the four cell strains CCRF-CEM, CEM/VP100, CEM/VP200 and CEM/VP500 by using two different synthetic polymer substrates, oligo(dT)·poly(dA) and oligo(dT)·poly(rA). A reduced DNA ligase activity was observed in the etoposide resistant substrains compared to cell line CCRF-CEM (Fig. 1). The reduction pattern in ligase activity was consistent, as demonstrated by simple linear regression analysis (Table 1). A statistically significant reduction was revealed between substrains CEM/VP100 and CEM/VP $\geq$ 200, but not between control and CEM/VP100, in two-tailed Students's *t*-test of the regression coefficients for the linear relationships. This pattern applied to oligo(dT)·poly(dA) ($P < 0.01$) as well as oligo(dT)·poly(rA) ($P < 0.05$), indicating reduction in DNA ligase activity in the two most resistant cell strains irrespective of used enzyme substrate, where all or only a subset of DNA ligases were measured.

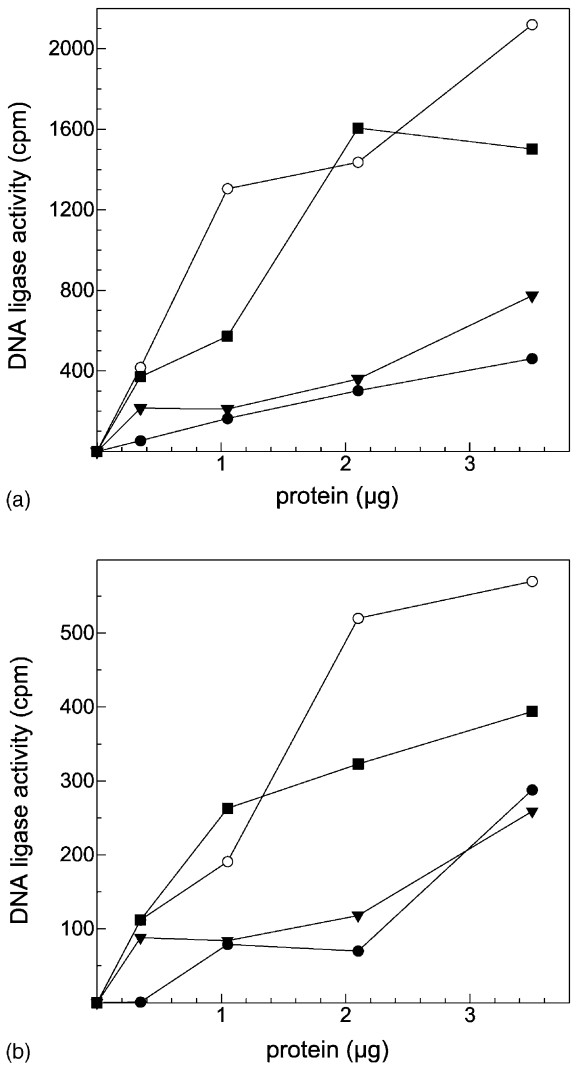

Fig. 1. DNA ligase activity in nuclear extract of acute lymphatic leukaemia cell line CCRF-CEM and substrains of increasing resistance to etoposide (CEM/VP100, CEM/VP200, CEM/VP500) using [^{32}P]oligo-(dT))·poly(dA) (panel a) and [^{32}P]oligo(dT) (panel b) as substrates. Symbols used: CCRF-CEM (open circles); CEM/VP100 (closed squares); CEM/VP200 (closed triangles); CEM/VP500 (closed circles).

To exclude that the reduction of the measured DNA ligase activity was not an effect of topo II levels in the resistant cell strains, we investigated if topo II was able to ligate the synthetic DNA ligase substrates [^{32}P]oligo(dT)·poly(dA) or [^{32}P]oligo(dT). No DNA ligating activity of the enzyme, up to the highest tested concentration (10 units) under the used test conditions was observed (data not shown).

3.2. DNA ligase–AMP complex formation

To investigate the initial, rate-limiting step of the DNA ligation process, the interaction between DNA ligase and ^{32}P-labelled ATP, to form a DNA ligase–AMP complex was determined. With equivalent amounts of protein, 7 μg nuclear extract of the four cell strains, the autoradiogram shows that the intensity of the bands corresponding to the

Table 1
Simple linear regression equations of DNA ligase activity, measured with two enzyme substrates, in CCRF-CEM and etoposide resistant substrains[a]

Cell strains	Regression lines	r^b
Oligo(dT)·poly(dA)		
CCRF-CEM	$y = 456.23x + 171.47$	0.913^{*}
CEM/VP100	$y = 564.07x + 265.70$	0.948^{*}
CEM/VP200	$y = 196.88x + 40.36$	0.967^{**}
CEM/VP500	$y = 131.77x + 11.71$	0.998^{***}
Oligo(dT)·poly(rA)		
CCRF-CEM	$y = 170.26x + 40.23$	0.956^{**}
CEM/VP100	$y = 104.37x + 72.27$	0.926^{*}
CEM/VP200	$y = 62.42x + 22.40$	0.942^{*}
CEM/VP500	$y = 77.31x - 20.64$	0.931^{*}

[a] Data derived from Fig. 1.
[b] Correlation coefficients.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

DNA ligase I-adenylate complex (125 kDa) and DNA ligase III–adenylate complex (100 kDa) did not decrease in the resistant cells but rather increased in comparison with the control (Fig. 2). An extra band, at 85–90 kDa, was observed in all the etoposide resistant cell strains. The experiments were repeated at least twice with reproducible results.

3.3. Western blot analysis of topo II and DNA ligase

Western blot analysis was used to measure the level of topo II, α and β, in the control cell line, CCRF-CEM and in the etoposide resistant cell strains, CEM/VP100, CEM/VP200, and CEM/VP500. Our results confirm data of reduced levels of both *iso*-forms of the topo II in the resistant cells [14]. The reduction of topo IIα and IIβ was proportional to the increased resistance (data not shown). Also, the amount of DNA ligase III in the nuclear extracts was investigated. Here a significant reduction of DNA ligase III protein, was observed in the most resistant cell strain, CEM/VP500, compared to the control CCRF-CEM (Fig. 3). The analysis was repeated with reproducible result. When analysing total cell extract from the cell strains no full size DNA ligase I protein was observed in any of them. Degradation products were observed in all cell strains but the pattern varied between the control and the resistant cell strains (Fig. 4). The amount of 85 kDa DNA ligase I fragment detected in the control cell line was only one third of the resistant cell strains. This was calculated as ratio between signal intensity of DNA ligase I bands in lanes and amount of the total protein in the lanes after staining of the membrane with Poncean S. Repeated experiments with cell extracts made from different preparations showed the same degradation pattern.

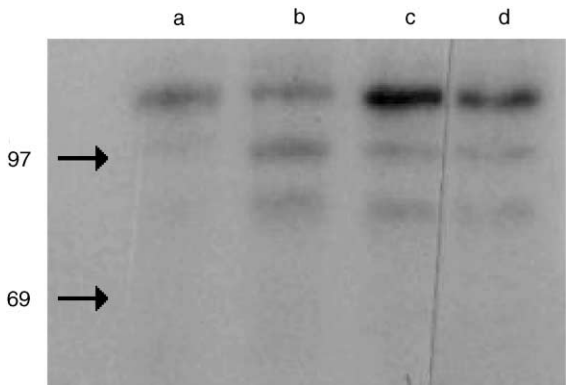

Fig. 2. Analysis of DNA ligase–adenylate complex formation from DNA ligases of nuclear extracts of acute lymphatic leukaemia cell line CCRF-CEM, cell strains thereof with increasing resistance to etoposide (CEM/VP100, CEM/VP200, CEM/VP500) and [^{32}P]ATP. Formed enzyme–adenylate complexes were separated by SDS-PAGE (7.5%) and analysed by autoradiography. An equivalent amount of protein (7 μg) was analysed from the cell strains. Lane a: CCRF-CEM; b: CEM/VP100; c: CEM/VP200; d: CEM/VP500. Molecular weight markers (kDa) are indicated.

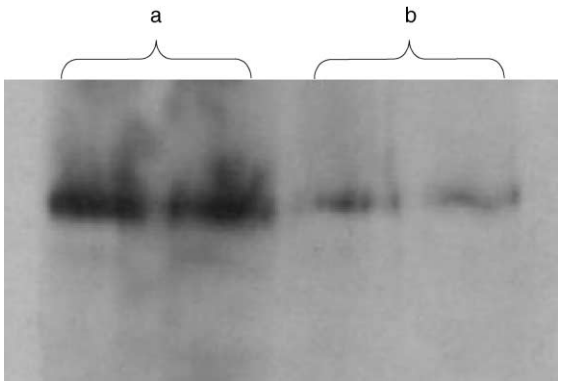

Fig. 3. Immunoblotting of DNA ligase III after separation of DNA ligases in cell extracts of CCRF-CEM and CEM/VP500 by gel electrophoresis. Equal aliquots of nuclear extract protein, 35 μg, from the two cell strains were separated according to their molecular mass by SDS-PAGE (7.5%) and analysed for DNA ligase III with monoclonal antibodies raised against the DNA ligase III. a: CCRF-CEM; b: CEM/VP500.

4. Discussion

Decreased levels of topo II protein or activity is reported to promote resistance to etoposide [2]. Since etoposide is preventing topo II religation of DNA strand breaks alternative pathways, to compensate for the deficiency and sealing the DNA breaks, are possible. DNA ligases are catalysing ligation of DNA strand breaks in a variety of processes in the cell, i.e. DNA replication, DNA repair and DNA recombination ([15], review). Today there are at least four DNA ligases reported, DNA ligase I–IV [15]. At least two of them, human DNA ligase I and rat DNA ligase III, are reported to act as topoisomerases by unwinding supercoiled DNA *in vitro* [16,17].

At investigation of DNA ligase activity in etoposide resistant cell strains reduced levels were observed in nuclear extracts of CEM/VP200 and CEM/VP500 when using the oligo(dT)·poly(dA) substrate, measuring all DNA ligases. The reduced levels of total DNA ligase activity could be suggested to reflect a decrease in the cell

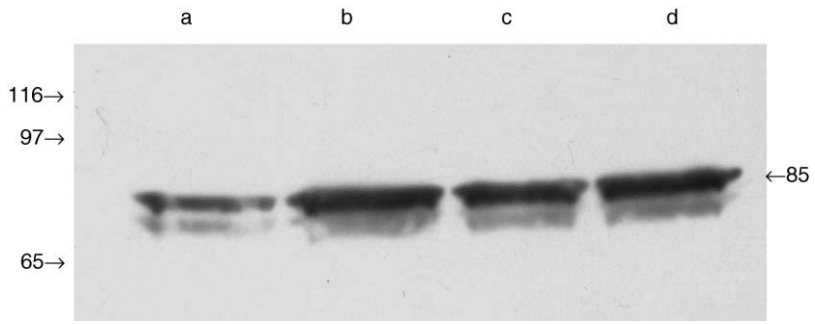

Fig. 4. Western blot analysis of DNA ligase I status. Proteins (30 mg) of total cell extracts were separated on 10% SDS polyacrylamide gel and assayed with DNA ligase I antibody (clone 7A12, Serotec Ltd.). Lane a: CCRF-CEM; b: CEM/VP100; c: CEM/VP200; d: CEM/VP500. Molecular weight markers (kDa) are indicated.

growth rate since DNA ligase I, the major DNA ligase, is known to be cell cycle regulated [18]. Measurements of the cell doubling time of the resistant cell strains, however, have not revealed any significant change in growth rate correlated to the resistance [14]. Also, at measurements of thymidine kinase enzyme activity, which is correlated to cells in S phase, no decrease in enzyme activity has been observed in the resistant strain CEM/VP500 compared to the control.[1] These data suggest that the decline in DNA ligase activity is not due to reduced cell proliferation or to fraction of cells in S phase. Neither, the observed reduced DNA ligase levels can be a result of enzyme inactivation by the drug since the half-life of the drug *in vitro* is short (6–8 hr) and the period between treatment and harvest was 2 weeks.

The increase, rather than decrease, of measured formation of DNA ligase I–adenylate complex in the nuclear extract (Fig. 2) of the resistant cell strains was unexpected in consideration of the observed reduced enzyme activity. This observation may reflect an increased expression of DNA ligase protein to compensate for the reduced DNA ligase activity. The enhanced amount of the 85 kDa DNA ligase I fragment in whole cell extracts of the resistant cell strains, compared to control (Fig. 4), supports this theory. The observation that the formation of DNA ligase I–adenylate complex was not decreased, even though the activity was reduced, indicates that the first rate limiting step in the DNA ligation reaction, i.e. the binding of AMP to the enzyme, is not affected. It rather suggests that a later step of the reaction is influenced.

At the use of synthetic oligo(dT)·poly(rA) measuring all DNA ligases but DNA ligase I, the activity was still reduced in the nuclear extract. This observation suggests that it cannot be excluded that also other DNA ligases (II, III or IV) are affected. DNA ligase II is today discussed either to be a form of DNA ligase III, an alternative gene splicing product, or more probably a proteolytic product of DNA ligase III [15]. Some increase in enzyme degradation fragments, measured as DNA ligase–adenylate complex, was detected in the most resistant cell strains as lower molecular mass products of the DNA ligases. At analysis of DNA ligase III by Western blot technique the levels of immunostained enzyme were substantially reduced in CEM/VP500. This result suggests a lower amount of DNA ligase III or an altered protein with lower affinity to the used antibody. The altered DNA ligase III observed is in accordance with the results by DeLange *et al.* that showed that vaccinia DNA ligase mutations conferred etoposide resistance [7].

Earlier observations have shown that anthracyclins, used as chemoterapeutic agents, are affecting the activity of DNA ligases, i.e. human DNA ligase I, rat DNA ligases I and III [19–21] and vaccinia virus DNA ligase [21] *in vitro*. The DNA binding property of the drugs indicated that their inhibitory effect mainly was via interactions with the DNA, rather than with the enzyme–adenylate complex formation step. The drug etoposide, however, did not inhibit the DNA ligation reaction by the DNA ligase tested, i.e. vaccinia virus DNA ligase, or caused accumulation of DNA–adenylate *in vitro* [22]. A direct inhibitory effect of etoposide therefore should not be expected in the performed experiments if some residual drug was present at cell harvest and the analysis of the DNA ligase activity.

Our data indicate that DNA ligases are significantly affected in the most resistant cell strains. Since etoposide exerts genotoxic activity, i.e. produces DNA strand breaks, micronuclei and mutations [23], an accumulation of mutations during the selection of the etoposide resistant cell strains is possible. Altered levels of protein phosphorylation is another factor that can influence the activity of DNA ligase I [24]. Mutations and altered phosphorylations, are also observed in the topo IIα gene and protein in the etoposide resistant cell strain CEM/VM1–5 [25]. Preliminary data from our laboratory on nuclear cell extracts of this cell strain and an additional etoposide resistant cell strain, CEM/V70-B1, originally established as resistant to teniposide [26] and merbarone [27], respectively, indicate reduced total DNA ligase activity. The levels were as low as in the most etoposide resistant substrain CEM/VP500.

In conclusion, the observation that DNA ligase activity, besides topo II, is reduced in the cell strains with high etoposide resistance suggests that not only affected topo II

[1] F. Albertioni, unpublished.

but also DNA ligases might contribute to the resistance. Since the present data suggest that there is no direct interaction between etoposide and DNA ligases further investigations are necessary to elucidate whether the altered DNA ligase in the etoposide resistant cell strains is coupled to the low levels of topo II protein via some common event, e.g. post-translational modification of proteins, altered gene expression, or an affected co-operation of the enzymes, or if the observed changes of the DNA ligases solely might contribute to the development of etoposide resistance. If the increase rather than decrease of DNA ligase–adenylate complex formation in the resistant strains is a cellular response to compensate for a reduced DNA ligase activity or reduced levels of topo II protein is still a task for further investigation. Further studies on a set of etoposide resistant primary cell lines of different origin, and cell lines with varying levels of different DNA ligases might give more details for the understanding of the complexity of drug resistance.

Acknowledgments

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